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Interleukin-33/ST2 signaling promotes production of interleukin-6 and interleukin-8 in systemic inflammation in cigarette smoke-induced chronic obstructive pulmonary disease mice

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ABSTRACT

Interleukin-33 is a newly described member of the interleukin-1 family. Recent research suggests that IL-33 is increased in lungs and plays a critical role in chronic airway inflammation in cigarette smoke-induced chronic obstructive pulmonary disease (COPD) mice. To determine the role of IL-33 in systemic inflammation, we induced COPD mice models by passive cigarette smoking and identified the IL-33 expression in bronchial endothelial cells and peripheral blood mononuclear cells (PBMCs) of them. After isolation, PBMCs were cultured and stimulated in vitro. We measured expressions of interleukin-6 and interleukin-8 in PBMCs in different groups. The expression of IL-33 in bronchial endothelial cells and PBMCs of COPD mice were highly expressed. Stimulated by cigarette smoke extract (CSE), the expression of IL-6 and IL-8 were induced and enhanced by IL-33. PBMCs of COPD mice produced more IL-6 and IL-8 stimulated by CSE and IL-33. Expression of IL-6 and IL-8 were decreased when stimulated by IL-33 together with soluble ST2. The mRNA production of ST2 in IL-33 stimulated PBMCs was increased. Being pretreated with several kinds of MAPK inhibitors, the secretions of IL-6 and IL-8 in PBMCs did not decrease except for the p38 MAPK inhibitor. We found that IL-33 could induce and enhance the expression of IL-6 and IL-8 in PBMCs of COPD mice via p38 MAPK pathway, and it is a promoter of the IL-6 and IL-8 production in systemic inflammation in COPD mice.

1. Introduction

Chronic obstructive pulmonary disease is a major worldwide and still increasing health problem [1], and it is characterized by incomplete reversible airflow obstruction associated with pulmonary inflammation involved in several kinds of inflammatory cells [2]. There are many kinds of inflammatory cells, cytokines and chemokines influencing injury and remodeling lungs and airways which are the characteristic changes of COPD [3]. Although the chronic irritants that trigger the inflammatory response of the airways in COPD were ceased, the inflammation still persists and the levels of cytokines in lungs and bloodstream remain abnormal in COPD patients even when they are in a stable phase of the disease. The persistence of systemic inflammation contributes to the development of systemic complications of COPD, including weight loss, skeletal muscle dysfunction, osteoporosis and atherosclerosis [4–6]. COPD patients have persisting chronic inflammation in their airways, and several studies have demonstrated that the systemic levels of inflammatory markers are much higher [7,8]. In other words, the chronic inflammation exhibiting in COPD patients is not only in lungs and airways, but also exists and influences other parts of the body.

Interleukin-33 (IL-33) is a cytokine of the interleukin-1 family, which also includes IL-1α/β and IL-18 [9], and singles via ST2 [10]. Most of the IL-1 family members play important roles in Th1 immune responses, but IL-33 is described as a promoter of Th2 immunity and systemic inflammation in vivo models and in vitro experiments [10]. In human and mice, IL-33 is expressed by innate cells, primarily epithelium and endothelium, and is released when they are stimulated by inflammation or necrosis [10,11]. Recent research suggests that IL-33 is a multi-effective

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cytokine that could induce complex immune responses in immunity and in diseases [12].

IL-33 is significantly increased in lung and plays an important role in respiratory disease [10,13]. It could induce airway inflammation, airway hyper-responsiveness and goblet cell metaplasia in allergen-naive mice, and aggravate asthma-like responses in allergen-exposed mice [13]. IL-33 plays a critical role in the airway inflammation in chronic respiratory disease, such as COPD, but whether or not it influences the production of IL-6 and IL-8 in systemic inflammation of COPD is unknown. In this study, we investigated the role of IL-33 in peripheral blood mononuclear cells (PBMCs) of cigarette smoke (CS) induced COPD mice models, and found that it could induce and enhance the expression of IL-6 and IL-8 in PBMCs of mice, especially for the COPD. We hypothesized that IL-33 participated in promoting production of IL-6 and IL-8 in systemic inflammation of COPD.

2. Materials and methods

2.1. Animal studies

All animal experiments were approved by the Huazhong University Animal Experiment Ethics Committee and were performed in accordance with the Regulations for Animal Experiments and Related Activities at Huazhong University.

Male Blab/c mice were obtained from Hubei Provincial Laboratory Animal Public Service Center, Wuhan, Hubei Province, China. Mice were housed in sterilized cages with filter tops in specific pathogen-free conditions at Tongji Medical College, Huazhong University of Science and Technology.

2.2. Cigarette smoke exposure and lung function measurement

The male mice were randomly divided into two groups, CS exposure group and Control group. The CS exposure mice were exposed to cigarette smoke passively in PAB-S200 Animal Passive Smoking Exposure System (BioLab Technology Co. Ltd., Beijing, China) for 8 months. We gave the CS group 10 research cigarettes inhaling for 3 h per day, and 5 continuous days for a week. Simultaneously, the controls were exposed to room air.

At the end, the lung function of all mice, including CS exposure and controls, were measured in the AniRes 2005 Lung Function system (BoiLab Technology Co. Ltd, Beijing, China). The FVC, $FEV_{0.1}$ and $FEV_{0.1}$/FVC were recorded. The CS exposure mice, in which $FEV_{0.1}$/FVC were below 0.7, were picked out as COPD group. Their blood were drawn off from inferior vena cava and lung were fixed with paraformaldehyde for Hematoxylin–Eosin and immunohistofluorescence for IL-33 staining, accompanied with controls.

2.3. PBMCs culture and stimulation

PBMCs were isolated from blood of all mice, including 6 COPD mice and 6 controls. After counted, PBMCs were seeded at a density of 10^5 cells/cm^2 and grown at 37 °C with 5% CO2 in 1640 medium with 10% heat-inactivated FBS. Then the stimulant was added to the well. The stimulus, including IL-33, soluble ST2 (R&D systems, USA), CSE (Murty Pharmaceuticals, Inc, Lexington, Kentucky, USA), and pharmacological inhibitors, were made up to different combinations in different groups. PBMCs were cultured with stimulus for 24 h. Then supernatants were collected and stored at −80 °C and PBMCs were gathered for RNA extraction. In some experiments, PBMCs were pretreated for 1 h with U-0126 (10 μM), SP-600125 (10 μM), SB-203580 (10 μM) before the stimulation of IL-33 (10 ng/ml), CSE (10 μg/ml), or both. The pharmacological inhibitors SB-203580 (a p38 MAPK inhibitor), U-0126 (a p42/p44 ERK inhibitor), SP-600125 (a JNK inhibitor) were purchased from Santa Cruz Biotechnology, TX, USA and dissolved in dimethyl sulfoxide (DMSO).

2.4. RNA extraction and real-time quantitative RT-PCR

Total cellular RNA was isolated from PBMCs after stimulation by using Trizol (Invitrogen, Carlsbad, CA) and reverse transcription was performed by using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Quantitative real-time PCR was performed by using the SYBR Green Real-time PCR Master Mix (Roche, Mannheim, Germany) with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers for Q-PCR were synthesized by Invitrogen and as follows: GADPH (forward, 5′-aactttggcattgtggaagg-3′; reverse, 5′-ggatgcagggatgat gttct-3′), IL-6 (forward, 5′-ccggagaggagacttcacag-3′; reverse, 5′-tcca cgatttcccagagaac-3′), CXCL1 (forward, 5′-tgcacccaaaccgaagtc-3′; reverse, 5′-gtcagaagccagcgttcacc-3′), CXCL2 (forward, 5′-aaagttt gccttgaccctgaa-3′; reverse, 5′-ctcagacagcgaggcacatc-3′), ST2 (forward, 5′-aggggaatgttgaactcacg-3′; reverse, 5′-caacccagcctaagggt-aca-3′). The cDNA fragments were denatured at 95 °C for 15 s, annealed and extended at 60 °C for 60 s for 40 cycles. Each sample was examined in triplicate and the amounts of the PCR products were normalized to that of GADPH which served as internal control.

2.5. IL-33, IL-6 and IL-8 cytokines measured by ELISA

ELISA experiments were conducted on serum and PBMCs culture medium supernatants, which were collected when the stimulation was completed. Supernatants were spun down for 10 min to ensure the absence of cells in suspension and stored as aliquots at −80 °C until use. The mouse IL-33 CytoSet (Invitrogen) was used to quantify IL-33 as the manufacturer's instructions. And the mouse IL-6 and IL-8 CytoSet (Invitrogen) were also used to quantify IL-6 and IL-8. Each sample was examined in triplicate.

2.6. Statistical analysis

The quantitative RT-PCR data and part of the ELISA are expressed as the medians, whereas data from experiments done on IL-33 serum levels and IL-6, IL-8 protein levels in supernatants are expressed as means ± SEMs. Unless specified, data are representative of the indicated number of subjects or independent experiments. Student's t tests were performed to analyze the differences between data obtained in different groups of subjects. Parts of the paired data were analyzed by using paired Student's t test. Values of $p < 0.05$ were considered as statistical significant difference.

3. Results

3.1. Expression of IL-33 is increased in bronchial endothelial cells and PBMCs of COPD mice

Recent studies by Qiu et al. have demonstrated that the expression levels of IL-33 were markedly enhanced in the lung tissue of mice inhaling CS [14]. In our study, we measured the IL-33 expression in bronchial endothelial cells and PBMCs in CS exposed mice and controls. The expression of IL-33 was shown in immunohistofluorescence staining and noted in the nucleus of endothelial cells. IL-33 staining intensity of COPD was obviously higher than that of controls, and a quantitative analysis of the IL-33 staining in bronchial endothelial cells area showed a 3.9-fold increase in COPD

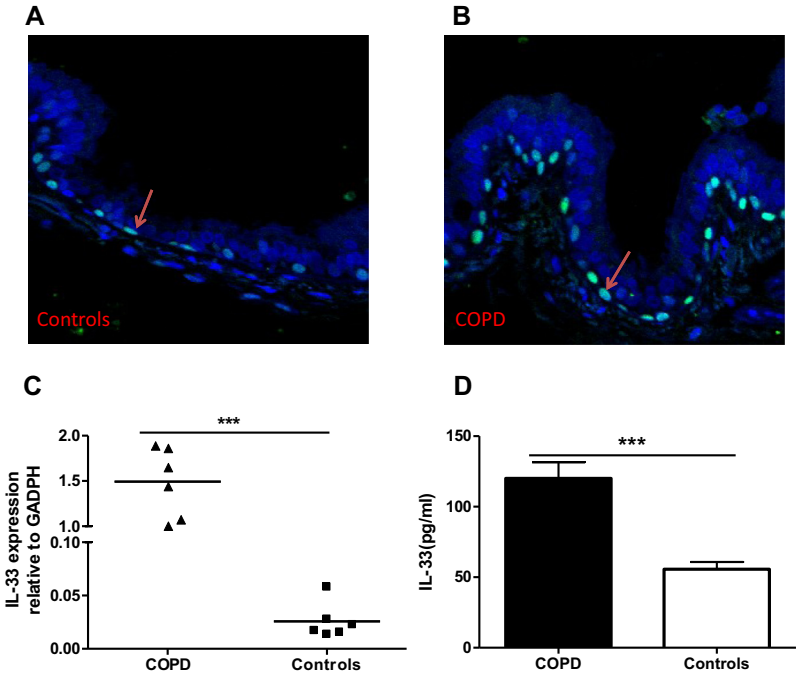

Fig. 1. Expression of IL-33 is increased in bronchial endothelial cells and PBMCs of COPD mice. The expression of IL-33 was shown in immunohistofluorescence staining in COPD mice (A) and controls (B). IL-33 staining in bronchial endothelial cells was showed in the nucleus and marked by red arrows. (C) The mRNA levels of IL-33 are expressed as relative to GADPH. Data for individual samples are presented as individual dots and the lines represent medians; (D) The protein levels of IL-33 secreted into the supernatants of PBMCs for all COPD mice ($n = 6$) and controls ($n = 6$) were observed. $^{***}p < 0.0001$; significantly different from the controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mice compared with controls (gray intensity, 51.3 ± 15.3 in COPD vs. 13.1 ± 3.8 in normal subjects) (Fig. 1A and B). The IL-33 mRNA expression in PBMCs of COPD mice was significantly higher than that controls as expressed relative to GADPH (Fig. 1C), the mean of IL-33 protein level in serum was higher in COPD mice than in controls (Fig. 1D).

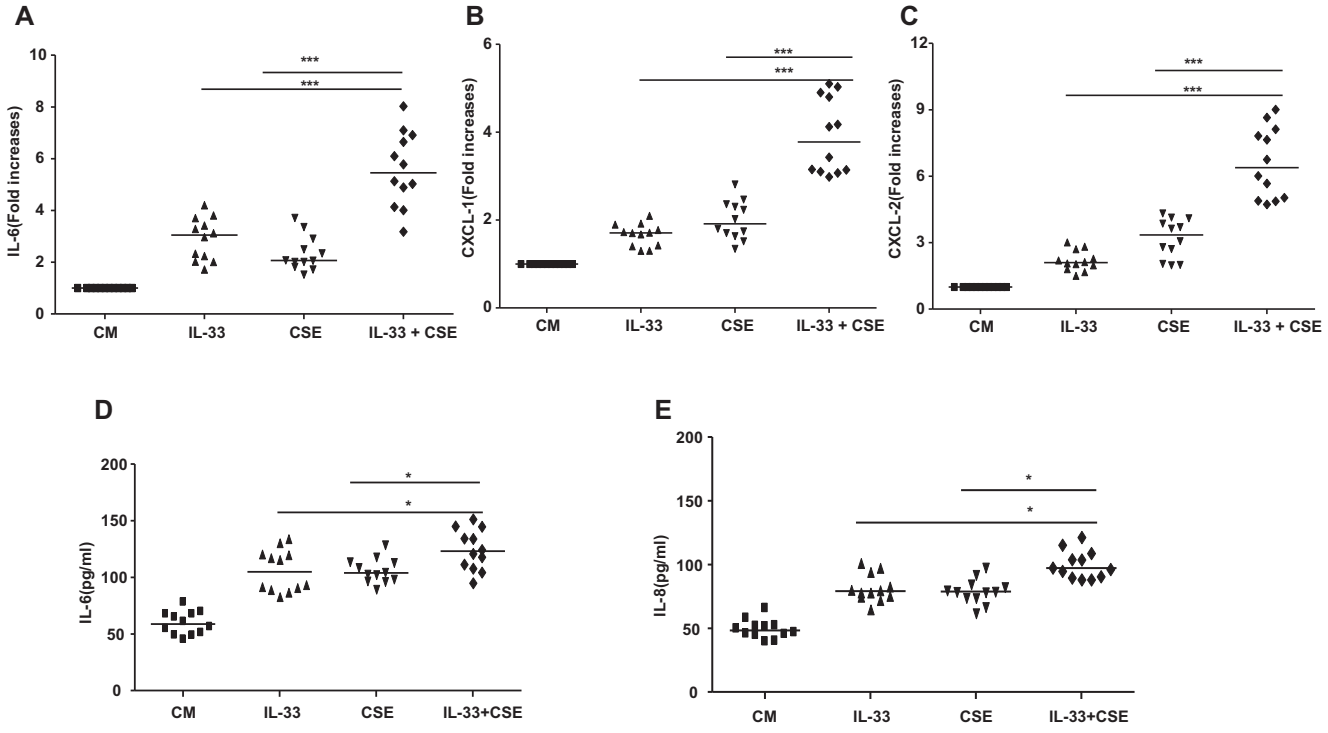

Fig. 2. TheIL-6 and IL-8 mRNA and protein expression are induced and enhanced by IL-33. (A), (B) and (C), the IL-6 and IL-8 mRNA expression were significantly increased by CSE (10 μg/ml) and IL-33 (10 ng/ml) stimulated together compared to separately for PBMCs in mice. (D) and (E), the changes of IL-6 and IL-8 protein expression were similar to their mRNA expression. $^{***}p < 0.0001$, represents statistically significant difference.

3.2. IL-33 induces and enhances the expression of IL-6 and IL-8 in PBMCs of mice

In parallel to the increased IL-33 expression in bronchial endothelial cells and PBMCs in COPD mice, we used IL-33 stimulating the PBMCs in vitro to determine the expression of pro-inflammatory cytokine IL-6 and chemokine IL-8, which have been reported as promoters of inflammation in COPD [15]. We stimulated the PBMCs in vitro by using IL-33 and CSE together or separately, and the concentration of IL-33 as stimulant was 10 ng/ml and CSE was 10 μg/ml. The mRNA expression of IL-6 (Fig. 2A) and IL-8 (Fig. 2B and C) were increased when stimulated by IL-33 and CSE together compared to separately. The protein expression of IL-6 (Fig. 2D) and IL-8 (Fig. 2E) also performed the same changes. The PBMCs stimulated by IL-33 and CSE together produced more mRNA of IL-6 and IL-8 and secreted more IL-6 and IL-8 into the supernatant.

3.3. The PBMCs of COPD mice are more sensitive to the stimulus of IL-33 and CSE

In COPD patients, serum concentrations of IL-6 and IL-8 are elevated [15] and they promote the progress of chronic inflammation. In our study, we stimulated the PBMCs of COPD mice and controls by CSE and IL-33 to determine the responses of PBMCs to the stimulus. PBMCs of COPD mice produced more mRNA and protein of IL-6 (Fig. 3A and D), compared to controls after being stimulated by IL-33 with or without CSE. Simultaneously, the mRNA and protein production of IL-8 in PBMCs of COPD mice were also elevated (Fig. 3B, C and E) after the stimulation. By the above paired comparison, COPD mice were more sensitive to the stimulus of IL-33 and CSE.

3.4. IL-33 increases the expression of IL-6 and IL-8 in PBMCs by binding to ST2

Cigarette smoke could induce IL-33 and ST2 expression in naive mice lung [14], and IL-33 playing its critical role in CS-induced airway inflammation signals via ST2 [14]. Our study focused on the changes of proinflammatory cytokine and chemokine in PBMCs of CS-induced COPD mice. We have already found that IL-33 acted as a promoter in producing IL-6 and IL-8 in PBMCs of COPD mice. The stimulus to the PBMCs of mice were adjusted to two paired groups, IL-33 with soluble ST2 compared to IL-33 only and IL-33 and CSE together with soluble ST2 compared to without it. The IL-6 and IL-8 protein expression in PBMCs decreased after the stimulation with soluble ST2 compared to that without it (Fig. 4B and C). Nevertheless the ST2 mRNA expression increased in the IL-33 stimulated PBMCs (Fig. 4A). Hence, IL-33 plays its role in promoting and enhancing the production of IL-6 and IL-8 by binding to ST2.

3.5. IL-6 and IL-8 released from PBMCs induced by IL-33 are via p38 MAPK pathway

Within the IL-1 family, IL-33 is found to be the unique one that is associated with the promotion of systemic Th2 immune

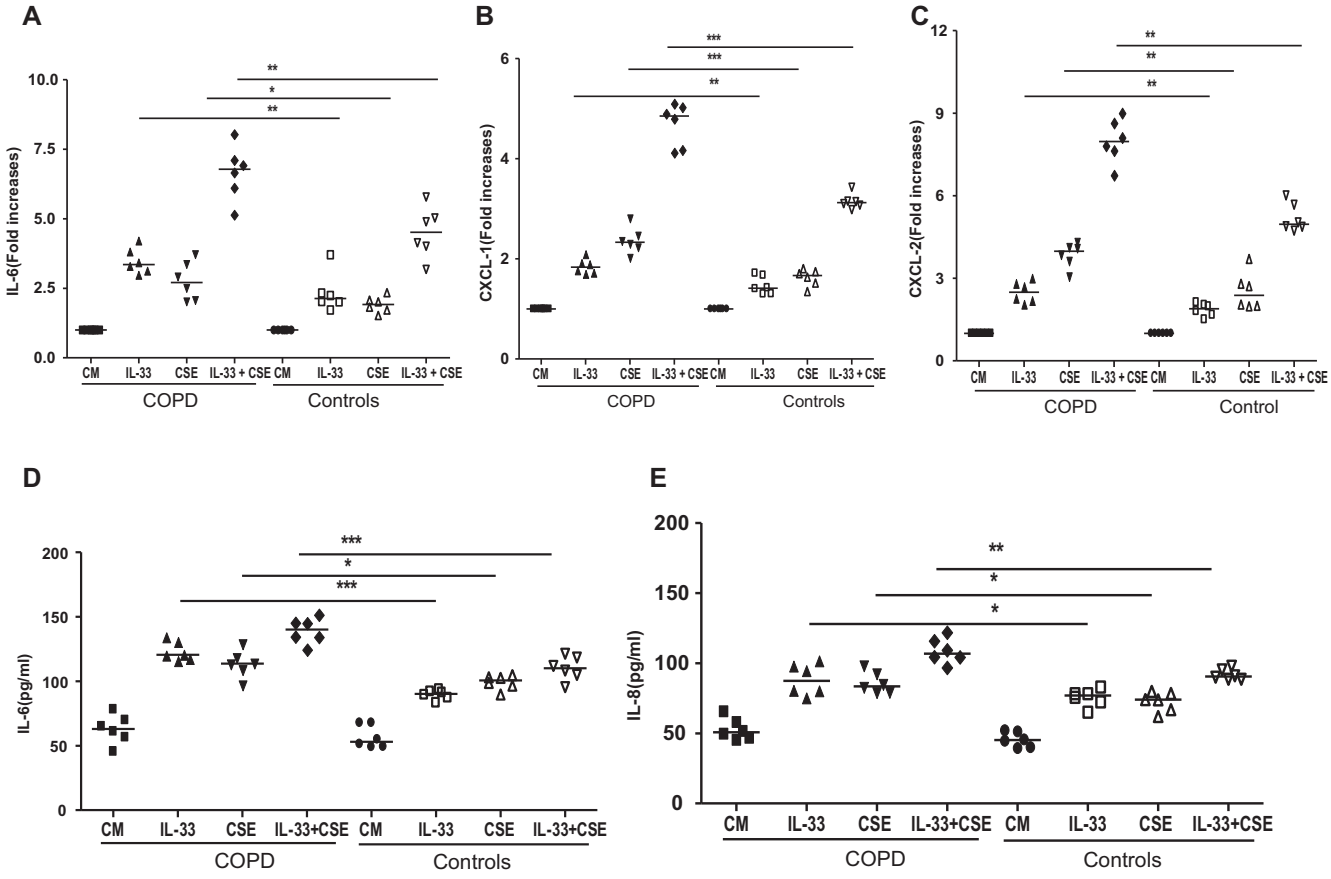

Fig. 3. PBMCs of COPD mice produce more IL-6 and IL-8 under stimulation. (A) IL-6 mRNA and (D) protein expression in PBMCs were enhanced in COPD mice compared to controls under the stimulation of IL-33 and CSE together or separately. (B), (C) and (E), mRNA and protein expression of IL-8 in PBMCs of COPD mice also increased much more under the stimulation. Data for individual samples are present as individual dots and the lines represent the medians. $^{*}p < 0.01$, $^{**}p < 0.001$, and $^{***}p < 0.0001$, represent statistical difference between the paired groups.

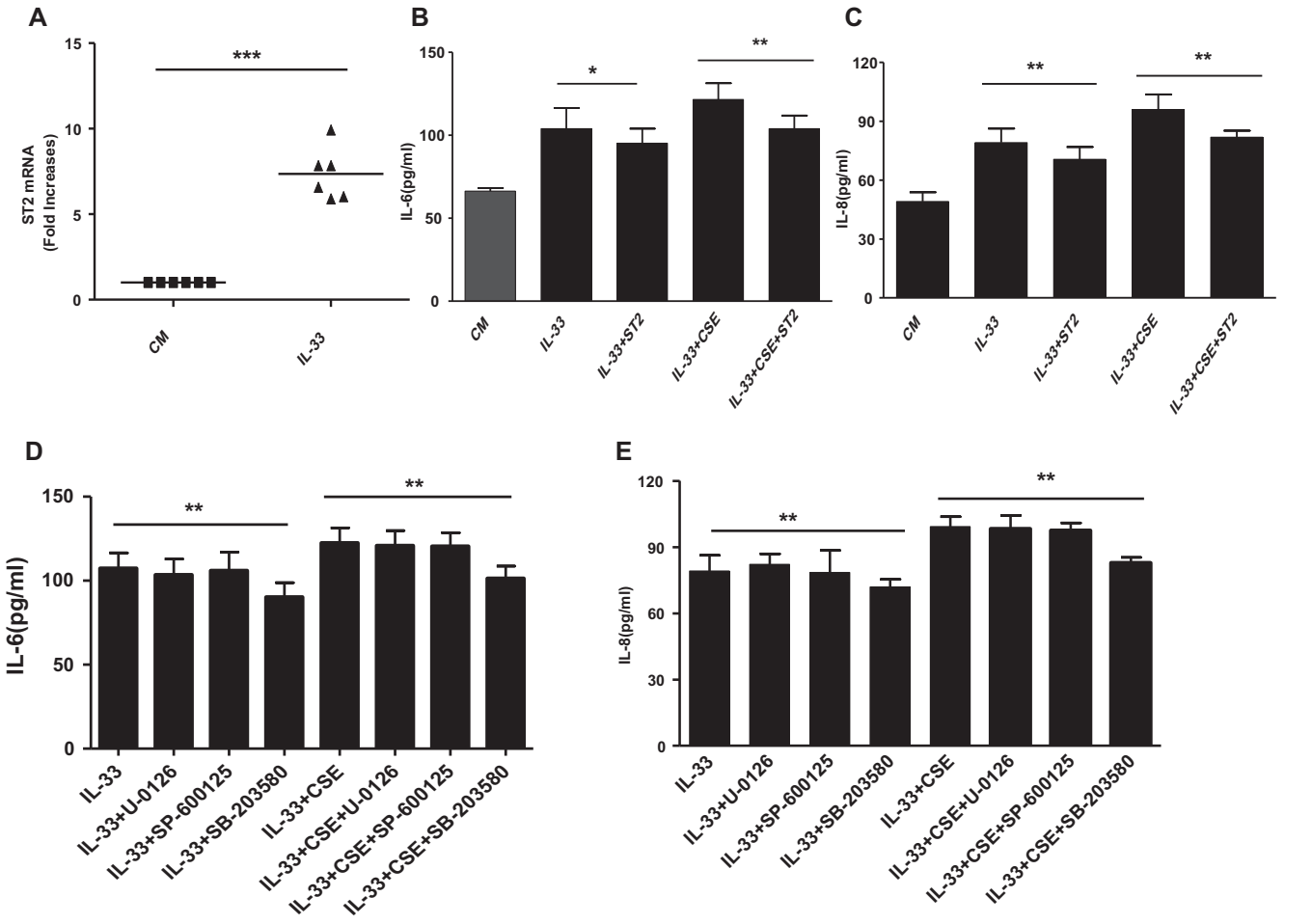

Fig. 4. The IL-33-mediated IL-6 and IL-8 release from PBMCs was binding to ST2 and abrogated by p38 MAPK inhibitors. (A) The PBMCs of mice stimulated by IL-33 produced more mRNA of ST2. The protein production of IL-6 (B) and IL-8 (C) in PBMCs under stimulation of IL-33 and CSE with the soluble ST2 were decreased compared to the stimulation without it. $^{*}p < 0.01$, $^{**}p < 0.001$, and $^{***}p < 0.0001$, represent statistical difference between the paired groups. The IL-6 (D) and IL-8 (E) secreted into the supernatants from the PBMCs of mice under the stimulation of IL-33 or together with CSE were abrogated by p38 MAPK inhibitors. $^{**}p < 0.001$, means were significantly different from the group without inhibitors.

responses until now [16]. After binding its receptor complex which consists of ST2 and IL-1 receptor accessory protein (IL-1RacP), IL-33 activates the mitogen-activated protein kinase (MAPK) pathway, including the extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinase (JNK), to achieve its effects on the promotion of Th2 inflammation [12,17]. Our study found that IL-33 was a promoter and enhancer of the IL-6 and IL-8 production in COPD mice. We pretreated the cells for 1 h with U-0126 (10 μM), SP-600125 (10 μM), SB-203580 (10 μM) separately before the 24 h stimulation with IL-33 (10 ng/ml) and CSE (10 μg/ml), or IL-33 only. IL-6 and IL-8 released from PBMCs that pretreated with SB-203580 significantly decreased after stimulation with IL-33 and IL-33 together with CSE. The other inhibitors, U-0126 and SP-600125, did not have the same effect. The levels of IL-6 and IL-8 released from PBMCs that pretreated with these inhibitors had no statistical difference compared to the controls (Fig. 4D and E). Thus we determined that the IL-33-mediated IL-6 and IL-8 releasing from PBMCs was via p38 MAPK pathway.

4. Discussion

The immunopathogenesis of chronic obstructive pulmonary disease is involved in the innate and adaptive immune system. There are lots of immune cells and cytokines participating in the characteristic changes of COPD, such as thickening of the small airway walls and emphysematous destruction, which cause irreversible airflow limitation. C Qiu's report showed that the expression levels of IL-33 and ST2 were enhanced in lung tissue of mice inhaling cigarette smoke [14]. Our data showed that IL-33 expression was increased in bronchial endothelial cells and PBMCs of COPD mice and that IL-33 could induce and enhance the expression of IL-6 and IL-8 in PBMCs of COPD mice, And that the IL-33 and ST2 were combined to enhance the production of pro-inflammatory cytokine IL-6 and chemokine IL-8 via p38 MAPK pathway. IL-6 and IL-8 are widely studied as the biomarkers of systemic inflammation in COPD patients and are proved to be associated with its progress and complications [15,18–20]. Our results suggested that IL-33 could promote the IL-6 and IL-8 production in PBMCs of CS-induced COPD mice.

COPD is chronic respiratory disease associated with important extra-pulmonary manifestations [21], and the pathogenesis of these systemic effects is mostly induced by the systemic inflammation [22]. Fabbri et al. suggested that COPD is a part of a chronic systemic inflammatory syndrome [23]. C Qiu's study reveals that IL-33 plays a critical role in the airway inflammation in CS-induced lung inflammation in mice [14], and the changes of immune system in COPD not only occur in lung, but also in other parts of the body. However there is no research focused on the role of IL-33 played in systemic inflammation of COPD. Our results

demonstrated that the bronchial endothelial cells and PBMCs of COPD mice produced more IL-33, and IL-33 induced and enhanced the expression of IL-6 and IL-8 in PBMCs of CS-induced COPD mice. IL-33 also played an important role in promoting the proinflammation cytokine and chemokine production in the body of COPD mice.

IL-33 is the unique IL-1 family member which is associated with the promotion of systemic Th2 response, and it is identified as the ligand for the orphan receptor ST2 [10]. IL-33/ST2 signaling is identified and expressed on most of the innate cells, such as macrophages, neutrophils, natural killer cells, and epithelial cells acting as the barrier of the body, and it is also selectively expressed on Th2 cells [24]. Jing Lin et al. reported that in human corneal epithelial cells IL-33/ST2 signaling was enhanced after exposure to IL-33 and IL-33 increased the production of pro-inflammatory cytokines (IL-6) and chemokines (IL-8) in both mRNA and protein levels [25]. There is no research reported about the function of IL-33 on PBMCs, especially in chronic obstructive disease. Hence, we stimulated the PBMCs of COPD mice and controls with IL-33 and CSE. The expression of IL-6 and IL-8 were increased and they were expressed higher in COPD compared to controls both in the stimulation of IL-33 and CSE separately or together. The PBMCs of COPD mice were much more sensitive to the stimulation, such as CSE and IL-33. IL-33 could enhance the CS-mediated inflammation to activate the expression of the pro-inflammatory cytokines and chemokines in vitro.

IL-33 is highly expressed in lung tissues and mediated its function by binding to ST2, and IL-33/ST2 signaling activates several signaling proteins, nuclear factor κB signaling and the mitogen-activated protein kinase (MAPK) pathway, which is mediated by the activation of the MAPKs extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinase (JNK) [12,17], and then activating signals could induce the expression of canonical IL-1 target genes, such as IL-6 and IL-8 [26]. In human corneal epithelial cells IL-33 promoted the production of pro-inflammatory cytokines and chemokines through ST2 and NF-κB signaling pathways [25]. Our results demonstrated that the expression of pro-inflammatory cytokine/chemokine, IL-6 and IL-8, decreased in the stimulation of IL-33 together with mouse soluble ST2, and the ST2 mRNA expression in PBMCs highly increased in the stimulation of IL-33. These suggested that IL-33 played its role in the PBMCs of COPD mice by binding to its receptor ST2. We measured the protein expression of IL-6 and IL-8 in PBMCs stimulated by IL-33 and pretreated with ERK inhibitor, JNK inhibitor and p38 MAPK inhibitor in each, and the expression in PBMCs pretreated with p38 MAPK inhibitor, SB-203580, decreased statistically. Our results showed that the overexpression of IL-6 and IL-8 stimulated by IL-33 in PBMCs was via p38 MAPK pathway. It was the first time for the investigation of the IL-33/ST2 signaling playing its central role via p38 MAPK pathway in promoting IL-6 and IL-8 production in systemic inflammation of CS-induced COPD mice.

Previous studies demonstrated that IL-33 played a critical role in the airway inflammation in respiratory disease, such as asthma and COPD [14,27]. C. Qiu et al. reported that IL-33 could stimulate the synthesis of further key pro-inflammatory cytokines, chemokines, mediators in the airways, and it triggers the airway inflammation in the mouse model of CS-mediated COPD [14]. These studies reveal that IL-33 played an important role in the airway inflammation, which is the local immune change. Thus it is very important in the immunopathogenesis of CS-mediated COPD, but the immune changes occur in the body are also critical. Our study focused on the changes of PBMCs in peripheral blood stream in mouse model of CS-mediated COPD. Our results indicated that IL-33/ST2 signaling could aggravate the overexpression of IL-6 and IL-8 in systemic inflammation via p38 MAPK pathway in COPD mice.

Conflict of interest

All authors have no conflicts of interest.

Sources of support

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.073.

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