Original Article
The mechanisms and significance of IL-33/ST2 in COPD

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Abstract: In order to investigate the mechanisms and significance of IL-33/ST2 in COPD, we recruited 90 subjects undergoing lung resection for solitary peripheral carcinoma in our hospital who were divided into three groups equally and randomly: non-smokers, smokers with normal lung function and COPD patients according to their clinical characteristics. When compared with the non-smokers, smokers with normal lung function, the majority of the COPD patients have a hobby of smoking and a weak lung function. The expression of IL-33, IL-1RAcP, ST2, IL-13, MUC5AC, Chi3l3, IL-25, TSLP, SCGB3A1, SFTPC, Krt5, Aq3, Trp63 and Ngfr were dramatically increased in the hTECs of COPD patients and all the genes were highly correlated with the IL-33/ST2 signal. When the IL-33/ST2 signal was interrupted, the expression pattern of these genes were reversed; the phosphorylation level of MEKKK, p38MAPK11, NIK1, IκB, TAK1, NF-κB, JNK1, 2, 3 and ERK1/2 were obviously elevated in the hTECs of COPD patients, all of which were involved in the pathogenesis of COPD but the exact correlation with IL-33/ST2 remains further investigation. These results demonstrated that the activity of IL-33/ST2 was enhanced dramatically in COPD patients, the canonical NF-κB signal pathway was activated when the IL-33/ST2 was enhanced in COPD, and the inflammatory cytokines associated with Th2 responses were secreted abundantly leading to pneumonia and bronchitis. So we propose that IL-33/ST2 is the gold standard diagnosis indicator of COPD and will provide the well-known knowledge for the clinical diagnosis and treatment.

Keywords: Chronic obstructive pulmonary disease (COPD), IL-33/ST2, canonical NF-κB signal, Th2 inflammatory response

Introduction

Chronic obstructive pulmonary disease is one kind of pneumonia caused by the inflammatory cytokines released by the inflammatory cells, it is the fourth cause of morbidity and mortality in the developed countries [1], and its typical characteristics includes incompletely irreversible airway constriction, progressive development and other symptoms mainly involved in the lung [2]. By now, the exact pathogenesis of COPD has not been fully demonstrated. The smoke exposure and infection lead to a series of abnormal inflammatory responses which correlate tightly with personal heterogeneity and environment factors. According to the statistics, smoke exposure is the leading cause of COPD [3, 4].

The COPD patients always have chronic bronchitis and lots of sputum, and it is incurable with the disease progression. Bronchitis reduces the lung function and it results in the high mortality of COPD. The main component of sputum is mucus which is composed by water, salt, mucins and other proteins. The mucins endow the sputum special viscoelasticity and rheology, the lower respiratory tract secrets various mucins including MUC2, MUC5AC, MUC5B, MUC6 and MUC8. MUC5AC and MUC5B are the leading two mucins in sputum, which are mainly secreted by the middle trachea [5].

The ratio of the components in mucins plays an important role in exerting mucosa's protection function. The abnormal immunology responses activate NF-κB signal pathway and enhance the

synthesis and secretion of MUC5AC, not the MUC5B. It is very crucial for the IL-33/ST2 signal in the progression and exacerbation of COPD but the detailed molecular mechanisms between IL-33/ST2 and NF-κB signal need further investigation.

IL-33 binds to a receptor complex composed of ST2 and IL-1RAcP. IL-1RAcP can enhance the affinity of IL-33 for ST2 [6, 7]. The IL-33/ST2 signal, which sheds bright light for novel therapies, is the therapeutic target for different kinds of human diseases such as cardiovascular disease and chronic pulmonary diseases such as COPD and asthma.

IL-33 enhances Th2 response in innate immunity, and its constitutive expression in the nucleus alarms intrinsically to unresolved inflammatory responses. And the nucleus localization of IL-33 is very important in immune homeostasis [8]. In COPD patients, IL-33 increases inflammatory responses caused by tissue or cell necrosis, and it acts as a deputy regulator of NF-κB target genes transcription. IL-33 can be secreted in the intracellular matrix, interacts with ST2 membrane receptor and IL-1RAcP, activates NF-κB signal pathway by inhibiting IκBα, IL-33 can activate MAPK pathway, enhance the phosphorylation level of ERK1/2, p38 and JNK1/2, but it is prone to regulate NF-κB signal pathway.

ST2, also named as T1, IL1RL1, DER4 or FIT-1, is one member of the IL-1 receptor superfamily, it contains four isoforms-ST2L, ST2V, ST2LV and sST2, among which sST2 acts as a secretion receptor and the other three as membrane receptors [9, 10]. The membrane receptors contain three extracellular domains, one trans-membrane domain and a SIR intracellular domain; sST2 lacks trans-membrane domain and intracellular domain. sST2 inhibits IL33/ST2 signal through ST2L, and the proportion of IL-33 and sST2 is the exquisite regulatory switch of IL-33/ST2 signal [6, 11-15]. In the periphery lymphocytes and epithelial cells of the bronchitis in COPD patients, the expression of IL-33 and sST2 are greatly increased, and sST2, whose concentration is negatively correlated with the prognosis [16], is the pathology indicator of COPD exacerbation. So IL-33/ST2 signal is a gold standard of COPD and acts as a therapeutic target in the improved strategies of treatment.

The immune cells expressing ST2 are as follows: Th2 lymphocytes, NK cells, NKT cells, mast cells, macrophages, monocytes, dendritic cells, epithelial cells, neutrophils and so forth [10, 14, 17, 18]. IL-33 binds to ST2 and activates downstream signals: the adaptors (e.g. MyD88, MAL or TIRAP, TRIF or TICAM1, TRAM or TIICAM2, SARM, et al.) interact with the receptor intracellular domain and IRAK; recruits TRAF6 [19]; activates MEKKK which activates p38MAPK, NIK1 which activates IκB, and TAK1 which activates NF-κB, JNK or p38MAPK. Besides, IL-33 indirectly regulates TNFα and IL-1β through binding to IL-1RAcP [20, 21], induces the expression of INF-γ, these demonstrates that in addition to mediate Th2 inflammatory response via ST2 receptor, IL-33 also exerts other functions not through ST2 receptor.

In COPD patients, the airway tract basal cells express high levels of IL-33 and maintain their multi-potency, and the release of IL-33 is regulated by ATP. IL-33/ST2 signal is the ideal target in treating COPD patients, and interrupting this signal is good to maintain the normal function of the airway tract mucosa immune system. In the severe COPD patients, IL-33 and IL-13 are increased, M2 macrophages aggregate, MUC-5AC is increased, and these phenomena indicate that Th2 inflammatory response is enhanced in COPD patients, but there are no effective strategies to inhibit Th2 response at present. IL-33 induced Th2 innate immune response regulates IL-13 expression through at least three mediators: TSLP, IL-25 and IL-33, these three mediators are expressed in epithelial cells, endothelial cells and different kinds of immune cells, and each mediator is necessary in Th2 inflammatory response, and is very crucial in the progression of COPD. These three mediators are highly expressed in COPD patients, but the exact biological mechanisms are not fully understood. Il-33 translocates into the nucleus, binds to H2A-H2B, tightens the chromatin structure and inhibits gene expression, but the physiological function of IL-33 as a nuclear factor needs further investigation.

In this study, we analyzed the potential role of IL-33/ST2 in the pathogenesis of COPD, and illustrated the activation of NF-κB and MAPK signal downstream of IL-33/ST2. In severe COPD patients, the tracheobronchial epithelial

Table 1. The Clinical Characteristics of non-smokers, smokers with normal lung function and COPD

Subjects	N	Age	Sex	Smoking history	Chronic bronchitis	FEV1% pred	FEV1/FVC%
Non-smokers	16	30~36	M:10/F:6	Lifelong non-smokers	No chronic bronchitis	126.4±8.5	89.1±5.5
Smokers with normal lung function	17	25~29	M:10/F:7	5 ex-smokers and 12 current smokers	6 with and 11 without chronic bronchitis	90.9±4.7	75.8±4.3
COPD	17	41~78	M:10/F:7	3 ex-smokers and 14 current smokers	12 with and 5 without chronic bronchitis	68.5±5.6	60.9±2.5

Definition of abbreviation: NLF, normal lung function; COPD, chronic obstructive pulmonary disease; FEV1, enforced expiratory volume in one second; FVC, forced vital capacity; FEV1% pred, FEV1 percent predicted. Data expressed as mean ± SEM.

cells (TECs) synthesis abundant IL-33, secrete it after cell damage, and increase the Th2 inflammatory response leading to differential gene expression in hTECs, epithelial lesions as well as pneumonia. The lung progenitor cells-serous cells, alveolar type 2 cells and basal cells-are the main source of IL-33 in the lung, and change the characteristics of the epithelial leading to the recruitment of inflammatory cells and overproduction of mucus in the sputum. When IL33/ST2 signal was interrupted by specific anti-IL-1RL1 mAb, the expression pattern of the lung progenitor cells was almost reversed. These phenomena demonstrated that IL-33/ST2 is the gold standard diagnosis indicator of COPD and paves the avenues to improved therapies.

Materials and methods

Collect the clinical characteristics and measure the lung function of all the subjects

All the subjects undergoing lung resection for solitary peripheral carcinoma were recruited from our hospital, all of them were agreed as participants and assigned written informed consent. The COPD patients are diagnosed based on the diagnosis guidelines of chronic obstructive pulmonary disease global initiative in 2006 [22] and the Global Initiative for COPD (GOLD) criteria and the FEV1/FVC is less than 70%. Our study was approved by the local Research Ethics Committee (Department of Respiratory Medicine, Fenghua People's Hospital). Collect the clinical information of all the participants, including sex, age, smoking history, bronchitis, FEV1%, FVC and FEV1/FVC%, and the detailed information is described in **Table 1**.

hTECs isolation and culture in vitro

The normal lung tissues of the subjects were obtained from lung surgery and the subpleural parenchyma of the lobe were excised as far away as possible from the tumor tissues. The lung tissues were minced into small pieces on ice using curved scissors. The lung slurry was mixed with 1 mg/mL Type IA-S collagenase and 50 U/mL DNase I (Sigma), then placed on a rotator at 37°C for one hour of digestion. The single cell suspension was passed through a 45 µm filter to remove large debris and undi-

gested tissues. Washed cells were suspended in UNC BEGM or UNC ALI medium, and centrifuged for 20 minutes, 1400 × g, room temperature, and the immune cells were removed. The adherent cells were sorted by anti-Epithelial Membrane Antigen (EMA) antibody (Ber-EP4) and anti-pan cytokeratin antibody (AE1-AE3) using FACS (two traditional epithelial markers). The sorted hTECs were cultured in UNC BEGM or UNC ALI medium.

Real-time PCR

Extract the total RNA from hTECs using 1 mL Trizol and reverse transcript into cDNA first strand. Test the relative gene expression level by real-time PCR. The detailed information of real-time PCR primers are indicated in the table as follows:

Gene	Primer Forward	Primer Reverse
IL33	GTGACGGTGTTGATGGTAAGAT	AGCTCCACAGAGTGTTCCTTG
IL-1RAcP	ACACTTCTGTGGTGTGTAGTGA	TGGTGTCTAGTCCCCAGTCAT
ST-2	CTCACCCGTTCCAACTCGAAG	TGGCAGACTTTTGACGCTTGA
IL-13	CCTCATGGCGCTTTTGTTGAC	TCTGGTTCTGGGTGATGTTGA
MUC5AC	TGCCCCTACAACAAGAACAAC	GGAACAGCACTGGGAGTAGTT
Chi3l3	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA
IL-25	CAGGTGGTTGCATTCTTGGC	GAGCCGGTTCAAGTCTCTGT
TSLP	ATGTTCGCCATGAAAACTAAGGC	GCGACGCCACAATCCTTGTA
SCGB3A1	TCCGCTCGTGCTTTCTTAGTG	GAGCCCTCTATGAGGTGGTTC
SFTPC	CACCTGAAACGCCTTCTTATCG	TTTCTGGCTCATGTGGAGACC
Krt5	CCAAGGTTGATGCACTGATGG	TGTCAGAGACATGCGTCTGC
Aq3	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
Trp63	GGACCAGCAGATTCAGAACGG	AGGACACGTCGAAACTGTGC
Ngfr	CCTACGGCTACTACCAGGATG	CACACGGTGTTCTGCTTGT

Detect the protein's phosphorylation level

The hTECs were cultured in plates and were lysate after chemical stimulation. The cell lysates were transferred into the detection plates, and added the HTRF detection reagents to measure the phosphorylation level of various proteins. All of the procedures were followed the guidelines illustrated in some published papers [23, 24].

Statistical analyses

The *t*-test and Pearson correlation test were used to analyze the results. All of the statistical analyses were performed by SPSS 19.0. Statistical results were expressed as the mean ± standard deviation (SD), *P* values<0.05 were considered statistically significant.
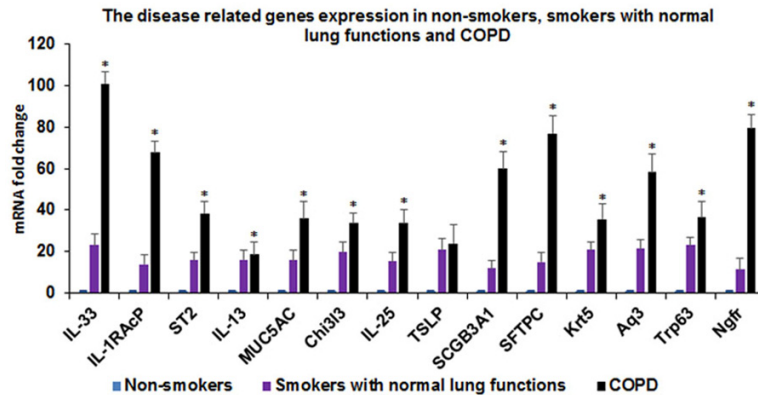
Figure 1. IL-33/ST2 signal and COPD related genes' expression pattern. In COPD patients, the hTECs showed an elevated expression of IL-33, IL-1RAcP, ST2, IL-13, MUC5AC, Chi3l3, IL-25, TSLP, SCGB3A1, SFTPC, Krt5, Aq3, Trp63 and Ngfr, and there was a significant difference when compared with the hTECs isolated from non-smokers and smokers with normal lung function. All of the genes were normalized to GAPDH and *P*<0.05 was accepted as statistical significance.

Results

Clinical information and lung function of non-smokers, smokers with normal lung function and COPD patients

As indicated by **Table 1**, all of the baseline clinical characteristics are shown in detail. Among the 17 COPD patients, the majority of them have a long smoking history, 3 are ex-smokers and 14 are current smokers, 12 with and 5 without chronic bronchitis, and all of the patients have a much weaker lung function indicated by the FEV1% pred (68.5±5.6) and FEV1/FVC% (60.9±2.5) when compared with the non-smokers and the smokers with normal lung function (**Table 1**).

IL-33/ST2 signal and COPD related genes' expression pattern

The human tracheobronchial epithelial cells (hTECs) were isolated from non-smokers, smoker with normal lung function and COPD patients after surgery. In order to investigate the effects of IL-33/ST2 in the pathogenesis of COPD, we measured the amount of IL-33, IL-1RAcP, ST2, IL-13, MUC5AC, Chi3l3, IL-25, TSLP, SCGB3A1, SFTPC, Krt5, Aq3, Trp63 and Ngfr in hTECs, among which IL-33, IL-1RAcP and ST2 are the ligands and receptors of the signal; IL-13 is regulated by this signal; MUC5AC is responsible for mucus production and Chi3l3 is the target gene of IL-13; IL-25, IL-33 and TSLP are three key

mediators associated with Th2 response; SCGB3A1 is one marker of the serous cells; SFTPC is one marker of alveolar type 2 cells; Krt5, Aq3, Trp63 and Ngfr are basal cell genes. Serous cell, alveolar cell and basal cell are the lung epithelial progenitor cells, smoke exposure and other airway infections result in the programmed gene expression changes of the epithelial cells, mucous cell and mucus formation induced by IL-13, as well as tracheobronchial lesions and Th2 inflammation consequently progressing into COPD. The real-time PCR demonstrated that in COPD patients, the hTECs showed an elevated expression of these genes, and there was a significant difference when compared with the hTECs isolated from non-smokers and smokers with normal lung function (**Figure 1**).
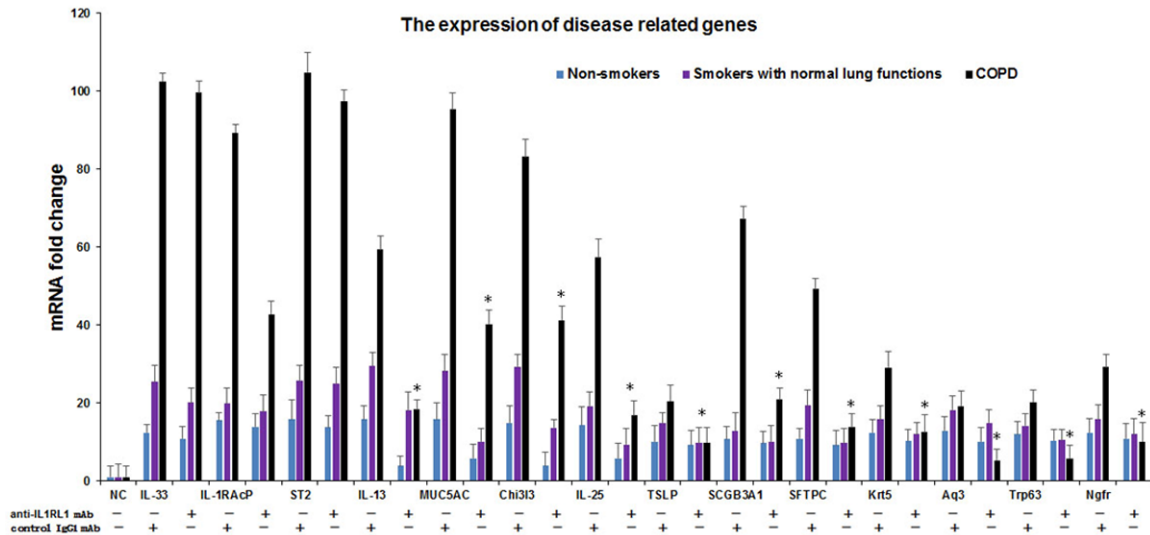
The genes' expression pattern after adding anti-IL-1RL1 mAb or control IgG1 mAb

To further investigate the crucial role of IL-33/ST2 in COPD, we inhibited this signal using the specific anti-IL-1RL1 mAb and IgG1 mAb was used as an isotope control. And then tested the expression levels of IL-33, IL-1RAcP, ST2, IL-13, MUC5AC, Chi3l3, IL-25, TSLP, SCGB3A1, SFTPC, Krt5, 14, 15, 17, Aq3, Trp63 and Ngfr performed by real-time PCR. These results demonstrated that when IL-33/ST2 was interrupted by anti-IL-1RL1 mAb, almost all the genes tested were down regulated except IL-33, IL-1RAcP and ST2 when compared with the isotope control (**Figure 2**).
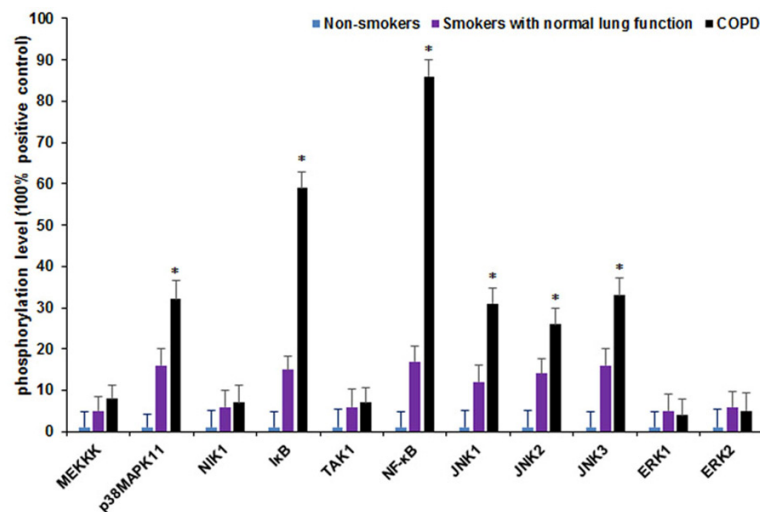
The proteins' phosphorylation level in non-smokers, smokers with normal lung function and COPD patients

When hTECs were isolated from the subjects, the phosphorylation level of MEKKK, p38-MAPK11, NIK1, IκB, TAK1, NF-κB, JNK1, 2, 3 and ERK1/2 were measured by HTRF phosphorylation detection kit. As illustrated by the results (**Figure 3**), p38MAPK11, IκB, NF-κB and JNK1, 2, 3 are presented with high phosphory-

Figure 2. The genes' expression pattern after adding anti-IL-1RL1 mAb or control IgG1 mAb. Specific anti-IL-1RL1 mAb or IgG1 mAb was used. And the expression levels of IL-33, IL-1RAcP, ST2, IL-13, MUC5AC, Chi3l3, IL-25, TSLP, SCGB3A1, SFTPC, Krt5, 14, 15, 17, Aq3, Trp63 and Ngfr were measured by real-time PCR (*P<0.05, all of the genes expression were normalized to NAPDH. Data is expressed as mean ± SD).

Figure 3. The proteins' phosphorylation level in non-smokers, smokers with normal lung function and COPD patients. The phosphorylation level of ME-KKK, p38MAPK11, NIK1, IκB, TAK1, NF-κB, JNK1, 2, 3 and ERK1/2 were measured by HTRF phosphorylation detection kit, and p38MAPK11, IκB, NF-κB as well as JNK1, 2, 3 are presented with high phosphorylation level. (*P<0.05, all of the genes expression were normalized to NAPDH. Data is expressed as mean ± SD).

lation level, indicating that MAPK, NF-κB and JNK are involved in the pathogenesis of COPD, but their crosstalk with IL-33/ST2 remains elusive.

Discussion

Chronic obstructive pulmonary disease (CO-PD) is an irreversible progressive chronic lung disorder which is usually associated with prolonged and enhanced inflammatory responses. Cigarette smoking and infection are the leading causes of COPD which is incurable until now [25].

The innate immunity can regulate the expression of IL-13 and the Th2 responses associated genes such as TSLP, IL-25 and IL-33. In the late COPD patients, the expression of IL-13 is accompanied by M2 macrophage differentiation and aggregation, the lung produces abundant MUC5AC mucins and bronchitis constricts leading to pneumonia and sputum increase.

IL-33/ST2 is the main signal that regulates IL-13 and is dramatically elevated in late severe COPD patients. IL-33 is a member of the IL-1 super-family, and is constitutively express a variety of cells such as epithelial cells, endothelial cells and fibroblasts. Mature IL-33 is released by cell damage or necrosis, so the immune system has to measure and regulate extracellular IL-33 which functions as an "alarmin" to signal cell damage. Clca3 and Muc5ac are involved in

mucosa synthesis; Alox12e, Arg1, Chit3l3/4, Mmp12 and Retnla are correlated with M2 macrophage differentiation. Muc5ac and Chi3l3 are target genes of IL-13 signal. But IL-33/ST2 and IL-13 cannot fully explain the pathology of COPD, so other signals and inflammatory cytokines should be paid lots of attention. IL-25 can regulate the expression of IL-13 and Muc5ac, but interrupting IL-25 signal has no effects on bronchitis, pneumonia and sputum increase [26].

Serous cells, and basal cells are three important cells in the lung [27-29]. SCGB1A1, SCGB3A1 and Cyp7b1 are the main markers of serous cells [29], SFTPC is the marker of alveolar type 2 cells [30] and the markers of basal cells include Krt5, Krt14, Krt15, Krt17, Aq3, Trp63 and Ngfr [31]. In COPD patients, the expression of IL-33, SCGB3A1, SFTPC, Krt5, Aq3, Trp63 and Ngfr is much higher, and IL-33 is mainly synthesized by endothelial and basal cells, secreted into the airway tract mucosa by epithelial cells, leading to a series of inflammatory responses and other lesions. Otherwise, the specific biological mechanisms of the markers' high expression regulated by IL-33/ST2 signal are not fully illustrated.

It has been reported that the concentration of soluble serum ST2 in slight and moderate COPD patients is twice higher than that of healthy smokers, and the soluble ST2 in serum plays an important role in tolerance of toxic environment, protects the over activated inflammation injury and is one kind of non-specific immune response [32].

It is necessary to look for one or more serum markers to detect the pathogenesis of COPD easily and simply. The ideal markers not only can distinguish between COPD stabilization with acute exacerbation period, but also can predict the severity of COPD, study the pathogenesis and epidemiology of COPD, and finally develop the novel treatment [33]. In 2011, the global strategy of chronic obstructive pulmonary disease firstly put the number of acute exacerbation of COPD into consideration as to determine the severity of patient evaluation index.

The immune response of COPD is primarily the Th1 response, and there are no differences in the concentration of IL-33 in sputum and serum when compared to healthy controls, and so is the ST2 in sputum. The ST2 is dramatically increased in serum of severe COPD patients in acute exacerbation period, and it returns to normal level in remission period, so we demonstrated that it is a serum marker to predict the pathogenesis of COPD.

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Disclosure of conflict of interest

None.

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