Original Article
Macrophage polarization involved the inflammation of chronic obstructive pulmonary disease by S1P/HDAC1 signaling

Min Zhang[1*], Ruoxuan Hei[2*], Zhou Zhou[3*], Wendi Xiao[1], Xi Liu[1], Yanwei Chen[1,4]

[1]Department of Pulmonary Critical Care Medicine, The 1st Affiliated Hospital of Shenzhen University, Shenzhen 518035, Guangdong, PR China; [2]Department of Clinical Diagnose, The Second Affiliated Hospital of The Air Force Military Medical University, No. 569 Xinsi Road, Xi'an 710038, Shaanxi, PR China; [3]Department of Pulmonary and Critical Care Medicine, Southern University of Science and Technology Hospital, Shenzhen 518102, Guangdong, PR China; [4]Department of Pulmonary and Critical Care Medicine, The Second Affiliated Hospital of The Air Force Military Medical University, No. 569 Xinsi Road, Xi'an 710038, Shaanxi, PR China. *Co-first authors.

Abstract: Globally, chronic obstructive pulmonary disease (COPD) is the cause of high morbidity and mortality, and constitutes a huge public health burden. Previous studies have reported that inflammation is closely related to COPD, but its potential mechanism is still unclear. Since the polarization of macrophages is involved in regulating inflammation, we assume that COPD changes the polarization of macrophages. To verify this, we investigated the relationship between the expression of S1PR1, HADC1, and inflammatory macrophages in COPD patients via flow cytometry, qRT-PCR, and western blot analysis. We found that macrophages of COPD individuals differentiated into M1 phenotype, and the expression of S1PR1 increased and HDAC1 decreased. S1PR1 also inhibits the expression of HDAC1, so S1PR1/HDAC1 signal regulates the polarization of macrophages. The results of the study put forward new ideas of the pathogenesis of COPD, and also proposed the possible treatment options.

Keywords: Chronic obstructive pulmonary disease, S1P, HDAC, macrophages, inflammation

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic lung disease characterized by chronic and persistent inflammation in the small airways and lung parenchyma, which leads to airway remodeling and parenchymal destruction [1]. It is not only a respiratory disease, but also a systemic disease involving systemic inflammatory manifestations [2, 3]. Systemic inflammation is characterized by an increase in circulating inflammatory cytokines even when the patient's condition is stable in COPD [4-6]. As we all know, macrophages and monocytes are widely involved in systemic inflammatory reactions, and also in pulmonary. However, their exact role in the pathogenesis of COPD have not been fully understood. It is widely accepted that macrophages may play an essential role in the pathogenesis of COPD [7-9].

Macrophages originate from two sources: the embryo macrophage progenitors and the bone marrow. In the adult, the bone marrow supplies monocytes to seed tissues and inflammatory lesions. Inflammatory monocytes are recruited from the blood, and bone marrow output of monocytes in the inflammatory process. Monocyte-derived macrophages and resident tissue macrophages often participate in inflammatory reactions, and sometimes some monocyte-derived macrophages convert into a cell as the resident tissue macrophages [10]. So monocyte-derived macrophages are more important to inflammation. Macrophages may be polarized at various stages of inflammatory reaction. Polarized macrophages can divide into two categories: classically activated macrophages (M1) and alternatively activated macrophages (M2) [8]. The M1/M2 type of macrophage polarization exerts important functions concerning inflammatory reactions [8]. Accu-

mulating evidence indicates that acute lung injury closely involves an extended or magnified M1 response and faulty M2 mediated repair. It was also reported that the imbalance in M1/M2 macrophage polarization may have adverse effects in inflammatory diseases, including COPD [11]. Especially M1 macrophages arise in inflammatory settings suggesting that M1 macrophage polarization is central to inflammatory reactions, which involved lung inflammation [8].

Sphingosine1-phosphate (S1P) serves as an important regulator of cell fate and immune responses in sphingolipid pathway [12, 13]. A great deal of progress has been made in deciphering its molecular mechanisms, previous studies have indicated that as an effective lipid medium, S1P regulates its biological activity by interacting with cell membrane receptors (S1P1-5) [14]. An extensive array of publications have elucidated that S1P can secrete its protein-coupled receptor into the extracellular environment, thence achieving the purpose of regulating cell-cell and cell-matrix interactions, and affecting cell migration, differentiation and survival [15]. Alteration of S1P signaling may leading to diseases, including hepatocellular carcinoma, atherosclerosis, Huntington's disease [16-18]. Sphingolipid molecules have been reported to have a protective effect on inflammatory lung diseases, and because of this, S1P may be relevant for treating of COPD and may be an effective therapeutic target for lung injury treatment [19, 20]. Therefore, polarization of macrophages might be of interest to probe the functions of S1P in the pathogenesis of COPD. Hait team found that S1P interacted specifically with histone deacetylases HDAC1 and HDAC2, and then diminish their enzyme activities. What's more, histone deacetylase (HDAC) is a direct intracellular target of S1P and link nuclear S1P to epigenetic regulation of gene expression, indicated that HDAC appear to be an intra-cellular target of S1P [21].

Histone Acetylase (HAT) and HDAC are intimately related to chromatin structure regulation and inflammatory gene expression. The HDAC activity and gene expression level in alveolar macrophages, lung tissue as well as peripheral blood mononuclear cells in COPD patients were lower than those in healthy controls [22-24]. HDACs are linked to macrophage activity.

Herein, we hypothesized that macrophage polarization involved the inflammation of COPD by S1P/HDAC1 signaling. To evidence our hypothesis, we examined the expression of S1PR1 and HDAC1 in monocyte-derived macrophages from controls and individuals with COPD, and by limiting and increasing signaling, assessed changes in cell activation.

Methods

Healthy volunteers and COPD patients

We have confirmed that all healthy volunteers and patients enrolled in this project have the freedom and right to obtain all relevant information about this project, and written consents were signed. The studies were conducted under the Declaration of Helsinki, International Conference on Harmonisation Good Clinical Practice Guidelines. The study was approved by the ethics committee of Shenzhen University located in Shenzhen, China (reference 20201208001). Criteria for recruiting patients with COPD included the following: 1) GOLD (2021) is used as the diagnostic basis of COPD in this study, and the ratio of forced expiratory volume (FEV1)/forced vital capacity (FVC) <0.7 post-bronchodilator, which proves the patients' airflow restriction; 2) between the ages of 40 and 70; 3) chest CT scan indicates abnormal emphysema; 4) all patients did not take any hormone drugs, theophylline, and other drugs. Exclusion criteria for recruiting patients with COPD included the following: 1) the presence of any other structural or functional lung disease, such as a past or current diagnosis of allergic rhinitis; 2) the presence of a respiratory infection or progressive exacerbation of COPD within 6 weeks before screening; 3) asthma; 4) peripheral blood eosinophil count >600 cell/mm^3; 5) hospitalization less than 12 weeks due to exacerbation of acute COPD; 6) the patient had a respiratory disease other than COPD; and 7) if it is necessary to inhale salbutamol due to unstable heart condition, it should stop 24 hours before each study visit. Patients are permitted to inhale corticosteroids and oral sustained-release theophylline. Oral or injectable corticosteroids are allowed in this study, and the intake dose of prednisone should be less than 10 mg/day, or 20 mg every two days. If oxygen therapy patients were treated for less than 15 hours a day within four weeks before

Table 1. Primers used for qPCR analysis

Gene	Primers sequences
HDAC1	5'-GGACTGTGTAGGCATCTTCTG-3'
	5'-CAATGACAGCTCCCACAAAG-3'
S1PR1	5'-TAGCACATGCAGCTTCTTTC-3'
	5'-TCCATGTAAACTGGGTGGTT-3'

Table 2. Basic information of patients involved in the present study

	Healthy group (n=5)	COPD group (n=7)	p
AGE (year)	61.60±10.38	59.00±6.53	0.604
HEIGHT (CM)	164.80±2.05	168.29±6.92	0.306
NORM_KG	63.86±1.34	66.14±4.52	0.305
BMI	22.74±4.77	21.07±4.38	0.542
BSA	1.67±0.16	1.67±0.17	0.997
FEV1/FVC	0.87±0.05	0.55±0.15	0.001
FVC%	2.56±0.75	3.83±0.72	0.015
FEV1%	2.24±0.70	2.12±0.84	0.805
FEF25_75	2.99±0.99	1.05±0.55	0.001
PIF	1.81±0.73	2.60±1.28	0.250

Healthy group and COPD patients did not take any hormone drugs, theophylline, and other drugs. COPD denotes chronic obstructive pulmonary disease; NORM denotes normal; BMI denotes body mass index; BSA denotes Body surface area; FVC denotes Forced vital capacity; FEV1 denotes forced expiratory volume in one second; FEF denotes forced expiratory flow; PIF denotes peak inspiratory flow.

screening, they can also be included in the study.

Healthy individuals with no history of chronic lung disease such as asthma, bronchiectasis, COPD were also included in this study.

Spirometry

Spirometry was measured in all patients following the standardized guidelines for ATS/ERS spirometry, and plethysmography was used to assess lung volume [25]. All experimental instruments are strictly carried out by the technicians with the relevant quality control program, technicians and doctors should confirm that all results are reliable. A spirometer (Vmax 229, Sensor-Medics, U.S.) was used for testing Flow-volume curves, FVC, relative FVC, FEV1, relative FEV1, and FEV1/FVC and the best volume of the three maneuvers was selected. Data was expressed as the percentage of predicted normal values. 200 µg salbutamol was

inhaled before lung function test (Glaxo Welcome, Chongqing, China).

Isolation of human monocytes from peripheral blood (PBMCs)

Patient derived venous blood (8 ml) was diluted 1:1 with 3.8% sodium citrate (Greiner Bio-One, Kremsmünster, Austria). The specific steps of PBMCs separation by density gradient centrifugation are described before [24]. The diluted venous blood (16 mL) was then added onto 8 ml LymphoPrep (density, 1.077 g/ml) (Ficoll, GE, China) and then centrifuged at room temperature at 1100 g for 30 min. The top liquid was discarded, the liquid layer containing PBMCs was absorbed, and the cells were washed with pre-cooled PBS once, and centrifuged at 300 g for 10 minutes. The use of ACK lysis buffer (Leagene, Beijing, China) is an optional step to reduce red blood cell contamination. The Countess Automatic Cell Counter (Invitrogen, Shanghai, China) measures cell viability and number. Cell viability is uniformly ≥95%.

Macrophage induction using lipopolysaccharide (LPS) and stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF)

PBMCs were isolated using Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS (Thermo Fisher Scientific, Shanghai, China) cultured at 5% CO_2 at 37°C for 3 hours after re-suspension. The medium was continuously stimulated with GM-CSF at a working concentration of 100 ng/mL for 14 days and medium was replaced every 2 days.

After that, cells were induced by 100 ng/mL LPS (Thermo Fisher Scientific, Shanghai, China) for 24 hours.

Flow cytometry

Flow cytometry was performed according to the previously published protocol [26]. Simply put, after treating PMBCS Cells with 0.25% trypsin (Sigma-Aldrich) without EDTA, the cell concentration was adjusted to $1×10^7$ cells/mL. Add 20 µL fluorescent dye conjugate antibody (1:1000) at room temperature for 30 minutes away from light. The antibodies used included phycothrin (PE)-Cy5-coupled CD86, fluorescein isothiocya-

A   Health (GM-CSF treatment)

B   COPD (GM-CSF treatment)
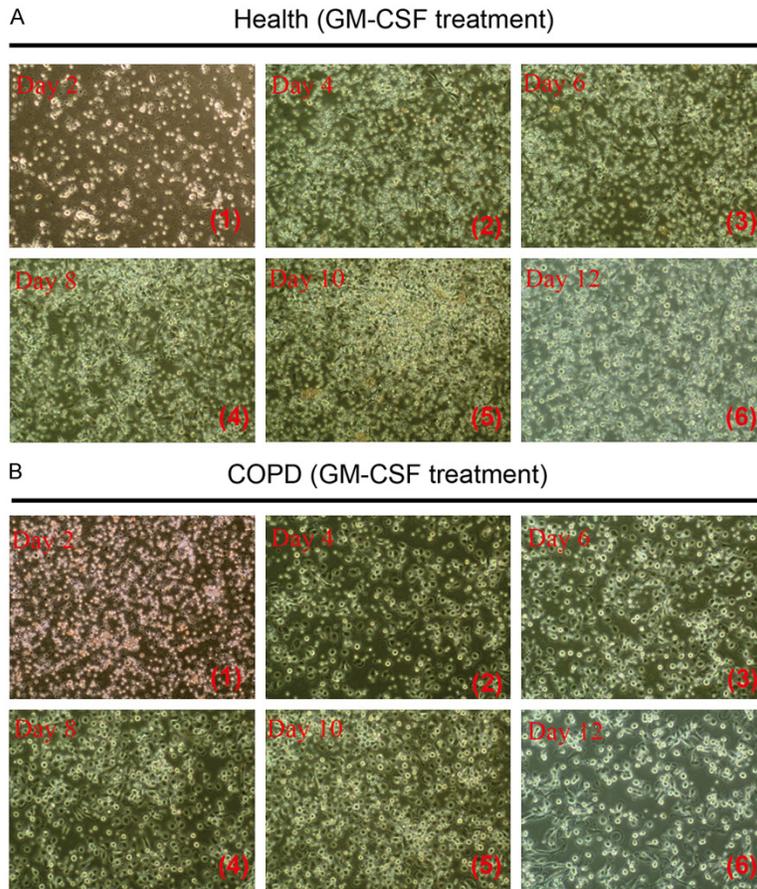
Figure 1. GM-CSF simulates the growth of macrophages from healthy (A, ×10) and COPD individuals (B, ×10) with time rhythm.

nate (FITC)-coupled CD80, isophycocyanin (APC)-coupled MHCII-DQ, and PE-coupled CD68, all purchased from eBioscience, San Diego, CA. After staining, analysis was performed using flow cytometry (BD FACS Calibur benchtop). FlowJo 7.6 software (FlowJo, BD, Ashland, USA) was used for data analysis.

Extraction, quantitation, and qualitative analysis of RNA

RNA was extracted from PBMCs using the Total RNA Extraction Kit (R6834-01, Omega, USA) following the manufacturer's instructions. The yield and purity of RNA were determined by a Nanodrop 2000 (Thermo Fisher Scientific, Shanghai, China). The qRT-PCR amplifications were carried out with LightCycler® 480 SYBR® Green I Mastermix (Roche, Shanghai, China). The qRT-PCR procedure follows the published protocol before [27]. All experiments were repeated independently. The comparative CT

method ($2^{-\Delta\Delta CT}$) was used to normalize target gene expression levels [28]. The primers used are listed in **Table 1**.

Plasmids and siRNAs transient transfection

Transient plasmid transfection is based on previous methods [29]. In short, when the cell density reached 60%~70%, the original medium was removed and 1 mL transfection medium was added. The ratio of plasmid or siRNA to Lipo3000 (Thermo Fisher Scientific, Shanghai, China) was set at 1:3, incubated at room temperature for 10 minutes, and then incubated in 12-well plates. The culture was then incubated in an incubator at 37°C and 5% CO_2, and after transfection for 3 hours, the culture medium was replaced and incubated overnight in a new incubator.

Western blot analysis

Protein samples were prepared in ice-cold RIPA buffer (P0013B, Beyotime Biotechnology, Shanghai, China) supplemented with Protease inhibitor cocktail for general use (100X, P1005, Beyotime Biotechnology, Shanghai, China). The cell proteins were separated on SDS-polyacrylamide gel and transferred to the PVDF membrane. After being blocked with nonfat milk, PVDF membranes were then incubated with the primary antibody (ab259902, Abcam) at 4°C overnight, and the next day treated with the enzymic secondary antibody (ab270144, Abcam) at room temperature for about 2 hours. The signal was displayed by enhanced chemiluminescence (P0018S, Beyotime Biotechnology, Shanghai, China) and the data was analyzed using ImageJ2 software.

Statistical analysis

All data are expressed as the means ± SE (Medians). Analysis of variance was performed with the use of the non-parametric Kruskal-
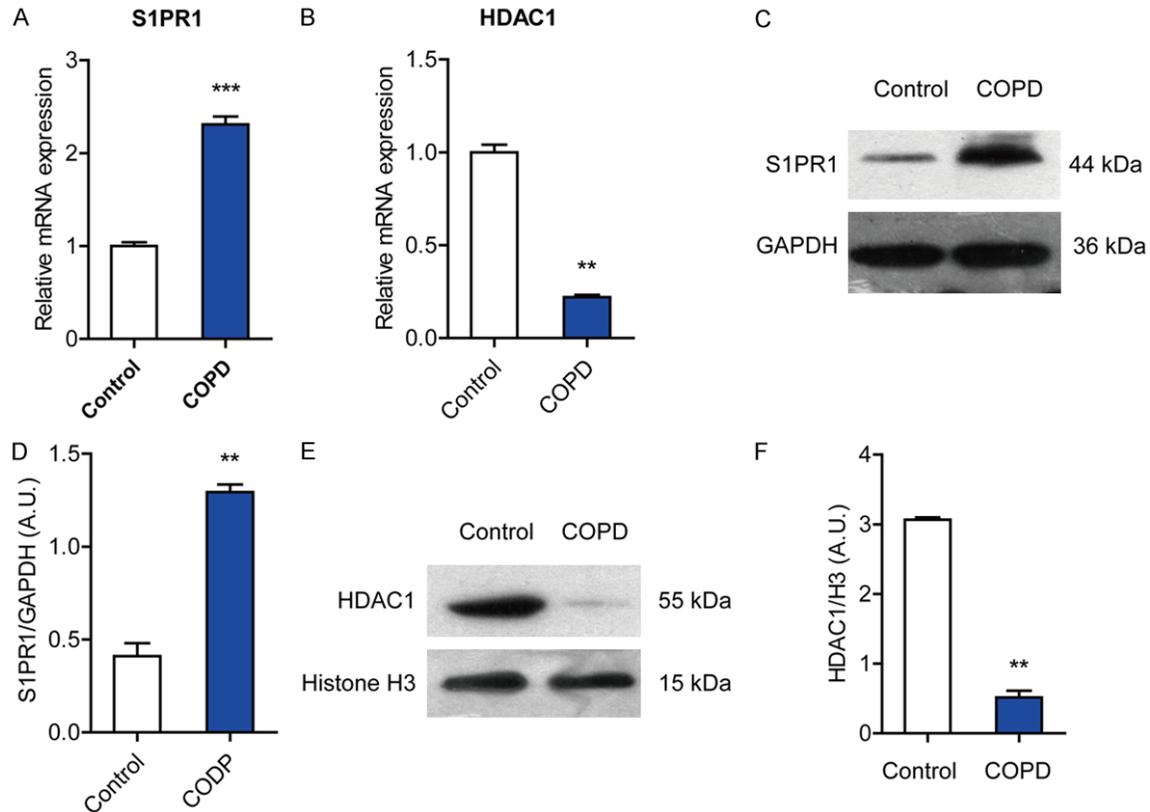
Figure 2. Expression of S1PR1 and HADC1. mRNA level of (A) *S1PR1* (***P<0.001*) and (B) *HDAC1* (***P<0.01*) in macrophages derived from COPD group (n=7) as compared with healthy controls (n=5). Protein level of (C, D) S1PR1 (***P<0.01*) and (E, F) HADC1 (***P<0.01*) in macrophages derived from COPD individuals (n=7) as compared with healthy controls (n=5).

Wallis test. When the result was significant, the Mann-Whitney U test was performed for comparisons between groups (SPSS software version 20.0). ANOVA be used for comparisons of multiple groups. P<0.05 was considered significant. All reported *p* values are two-sided.
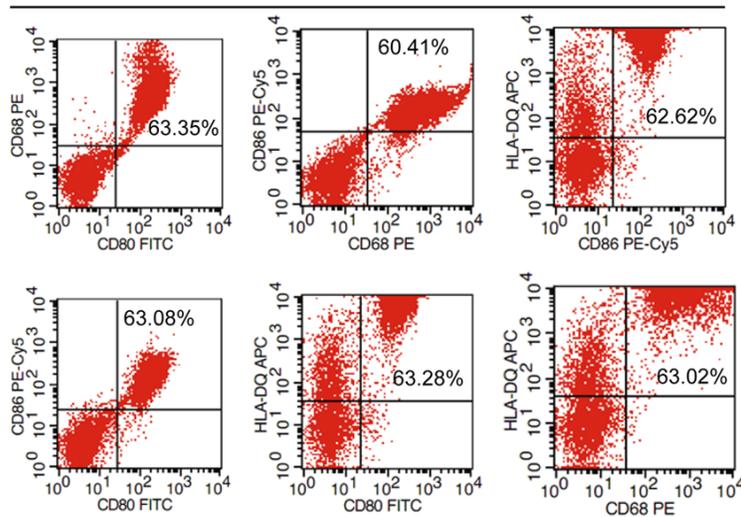
Results

Patient characteristics

Five healthy controls and seven COPD patients involved in the present study (**Table 2**). The Characteristics include age, height, BMI, BSA, FEV1/FVC, FVC, FEV1, FEF25_75 and PIK between COPD cases and controls. It was found that the difference between COPD and control was significant regarding FEV1/FVC (P=0.01), FVC% (P=0.015), and FEF25_75 (0.001). Other characteristics including age,

height, BMI, BSA, BMI, BSA, FEV1 and PIK were insignificant.

GM-CSF/LPS macrophage stimulation and S1PR1 and HDAC1 expression in healthy and COPD patients.

GM-CSF is widely used to stimulate macrophage differentiation (**Figure 1**). After stimulating healthy and COPD patient-derived PBMCs with 100 ng/mL GM-CSF, qPCR results showed a more than 2-fold increase in S1PR1 mRNA levels in COPD patients compared with healthy individuals (**Figure 2A**, *P<0.001*, n=7), and conversely, a lower HDAC1 mRNA level (**Figure 2B**, P<0.01, n=7). Similar to the changing trend of transcripts, the level of SIRP1 protein in macrophages of COPD patients was significantly higher (**Figure 2C**, **2D**, *P<0.01*, n=5), but the level of HDAC1 protein was decreased (**Figure 2E**, **2F**, *P<0.01*, n=7). M1 Macrophage polarization in the healthy and COPD groups.

A Health

B COPD
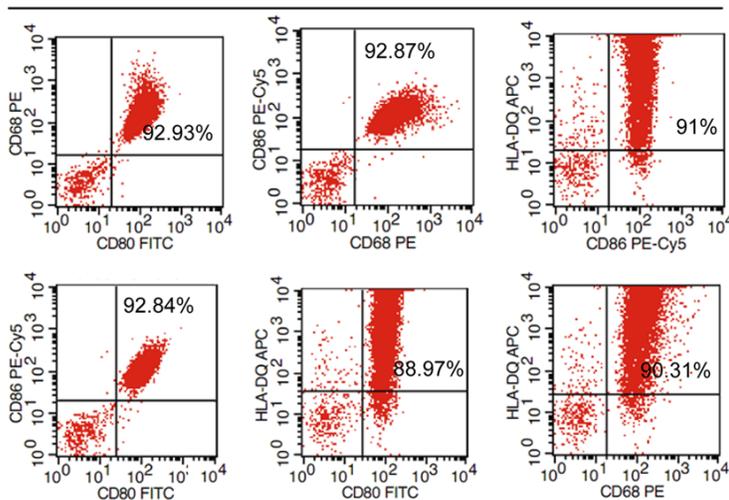
Figure 3. FACS analysis of macrophage polarization in cells. A. Representative results of the number of CD68/CD80, CD86/CD68, MHCII/CD86, CD86/CD80, MHCII/CD80, and MHCII/CD68 double-positive cells in samples from healthy controls. B. Representative results of samples from COPD individuals.

M1 macrophage polarization

To characterize macrophage phenotype, flow cytometry was carried out for detecting ratios of antigens CD68/CD80, CD86/CD68, MHCII/CD86, CD86/CD80, MHCII/CD80, and MHCII/CD68 [30]. Interestingly, CD68/CD80, CD86/CD68, MHCII/CD86, CD86/CD80, MHCII/CD80, and MHCII/CD68 double-positive cells (M1 macrophage cells) were significantly enhanced in macrophages from COPD individuals by comparison with the healthy group (**Figure 3**) [31].

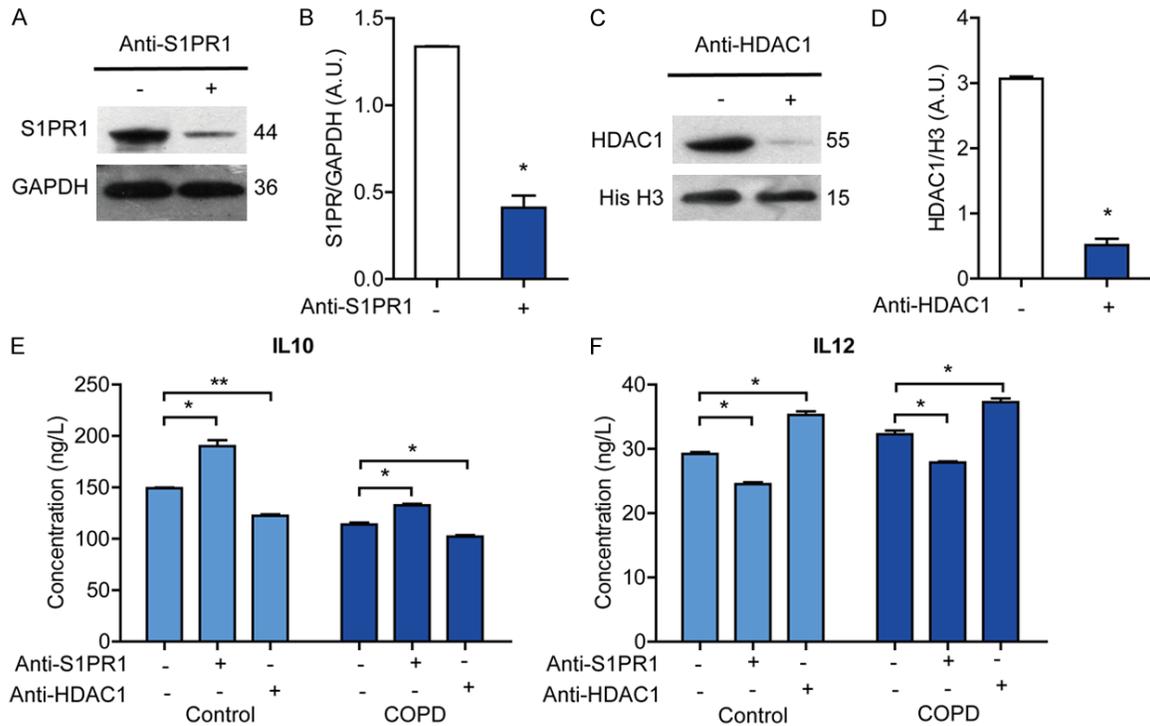*Effect of S1PR1 and HDAC1 inhibition on macrophage polarization*

At present, the effect of S1PR1 and HDAC1 on macrophage polarization is not clear. In order to understand the relationship between them, the expression level of S1PR1 and HDAC1 in macrophages after treated with siRNA was significantly blocked (**Figure 4A-D**). In addition, the expression of S1PR1 was negatively correlated with the production of IL-10 in the two groups of cells, and the production of IL-10 decreased with the reduction of HDAC1 expression (**Figure 4E**). Moreover, S1PR1 significantly reduced IL-12 levels, while knocking down HDAC1 increased IL-12 production in macrophages of healthy and COPD individuals (**Figure 4F**). Interestingly, there were apparent differences between healthy people and those with COPD.

To further confirm these results, macrophages from healthy controls were treated with FTY720 (S1P agonist). Similarly, limiting S1PR1 up-regulated IL-10-positive cells, undermined the number of IL-12-positive cells (**Figure 5A**, **5B**), as measured by flow cytometry. In addition, the inhibition of S1PR1 by MK571 resulted in increasing number of IL-10 positive cells and decreased IL-12 positive cells (**Figure 5C**).

S1PR1 up-regulate the expression of HDAC1

In order to study the relationship between S1PR1 and HDAC1, S1PR1 overexpression plasmid was constructed and transfected into macrophages originated from COPD subjects. Overexpression of S1PR1 significantly suppressed levels of nuclear and cytoplasmic histone acetyltransferase (HAT) ($P<0.05$, n=7,

Figure 4. Effect of the inhibition of S1PR1 and HDAC1 on macrophage polarization. A, B. The Western blot images show the knock-down efficiency of S1PR1 and HDAC1 (*P<0.05, n=5). C, D. Western blot assay used to determine the effect of the siHDAC1 on macrophages (*P<0.05, n=5). E. The concentration of IL-10 was detected in S1PR1 and HADC1 knockdown group respectively (*P<0.05, **P<0.01, n=5). F. IL-12 concentrations were also measured after S1PR1 and HADC1 knockdown (*P<0.05, n=5).

Figure 6A, 6B). Consistent with these data, overexpression of S1PR1 significantly suppressed the HDAC1 concentration in the nucleus and the cytoplasm (P<0.05, n=7, **Figure 6C, 6D**), it dramatically decreased IL-10 production (P<0.05, n=7, **Figure 6E**), but significantly increased IL-12 (P<0.05, n=7, **Figure 6F**) in macrophages from COPD individuals.

Discussion

COPD is a kind of respiratory disease which due to long-term exposure to noxious particles or gases, patients often show continuous respiratory symptoms and airflow limitation [32]. In addition to environmental pollution and smoking, genetic mutations are also one of the risk factors that have been in-depth explored for COPD, such as gene FAM13A, HHIP as well as HTR4 [33-37]. Besides, recent publications have reported that gene S1P and HDAC1 are widely differentially expressed in COPD patients and have the potential to become novel therapeutic molecular targets, so further exploration of their deep molecular mechanisms is needed

[22, 38]. While S1P, via S1PR1, is implicated in immunological, cardiovascular, and neurological processes [39]. The universal expression of S1P and S1PR1 are proved, and is associated with immunity [40]. Moreover, S1P positively correlated with the release of pro-inflammatory cytokines by regulating S1PR1 [41]. S1PR1 family members, including S1PR12 and S1PR13, are involved in bone marrow-derived macrophage motility [42]. Thus, our attention was particularly drawn to elucidating regulatory networks between S1P and COPD. Interestingly, we found that S1PR1 was highly expressed. HDAC1 is involved in innate and adaptive immunity as a deacetylating enzyme [43]. HDACs, including HDAC1, participate in the regulation of major histocompatibility complex (MHC) class I related genes and deacetylate the promoter of MHC class I [44]. And we found that HDAC1 is lowly expressed in COPD patients. We also confirmed that CD68/CD80, CD86/CD68, and CD86/CD80 double-positive cells were increased if cells were derived from COPD patients as compared to healthy subjects. These data emphasize that COPD derived mac-
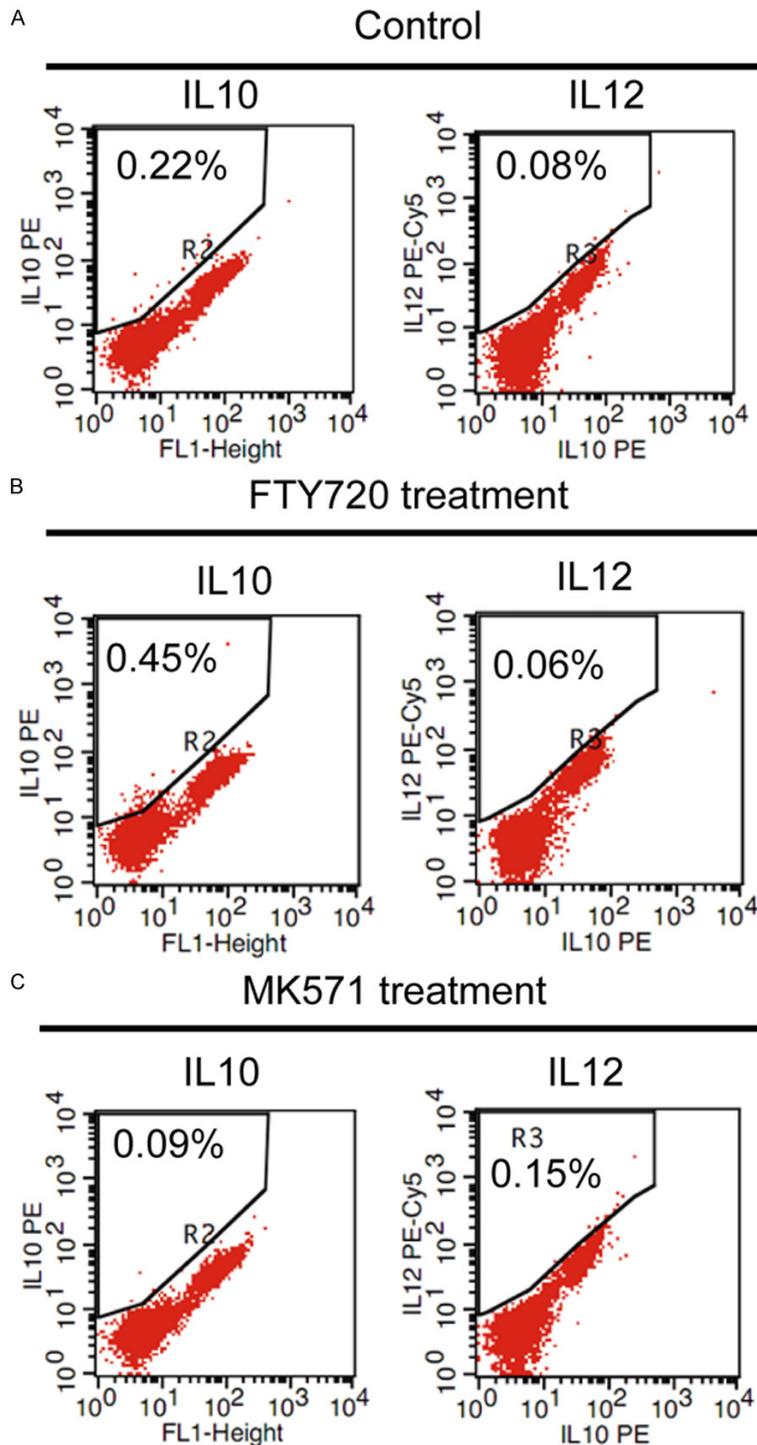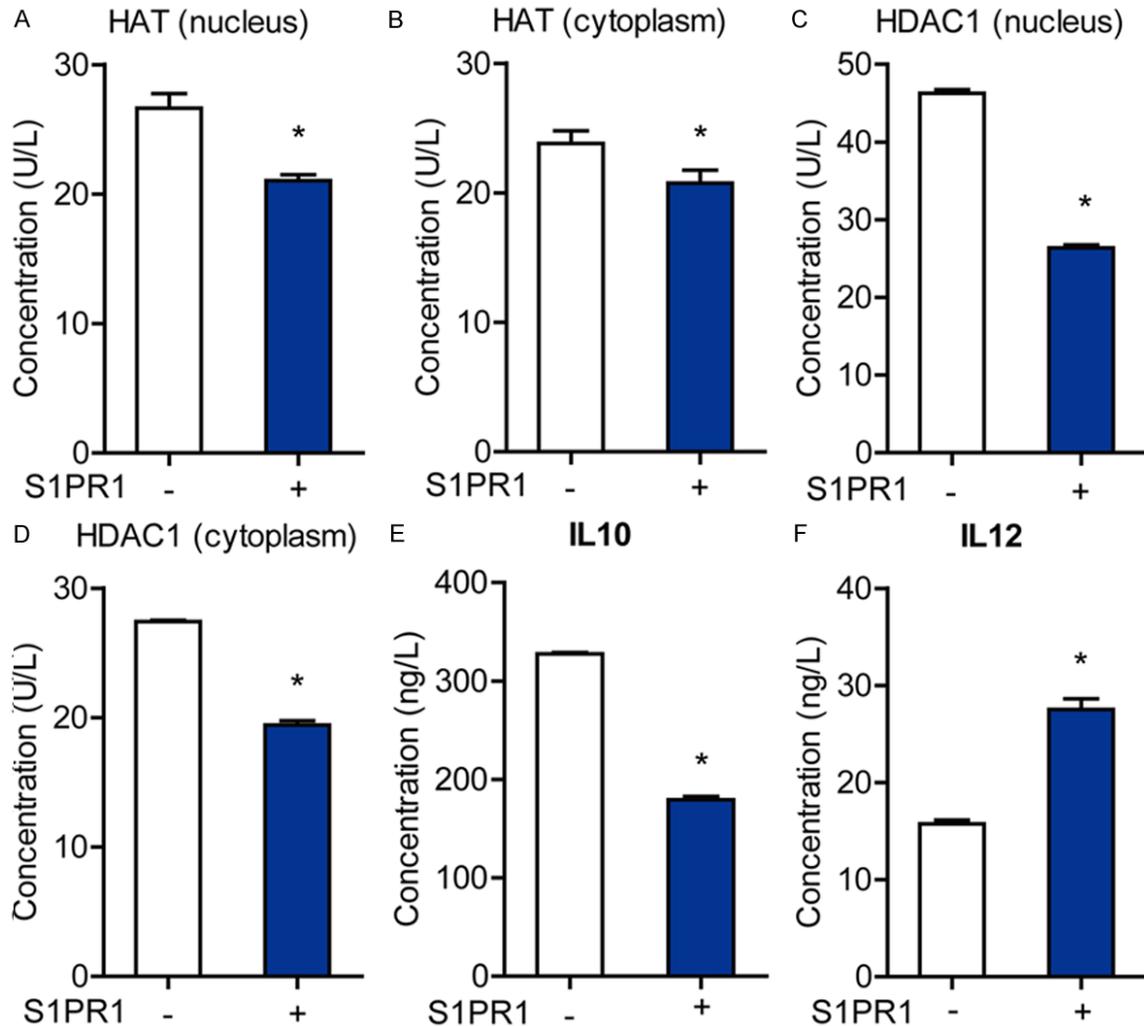
Figure 5. The number of IL-10- and IL-12-positive cells in the macrophages that received no treatment. (A) and in the groups that were treated with S1PR1 inhibitors, FTY720 (B) and MK571 (C).

macrophages (M1) and alternatively activated macrophages (M2) [46]. M1 macrophages boost the release of proinflammatory cytokines, including TNF-α, IL-12, and IL-1. Inversely, M2 macrophages produce anti-inflammatory cytokines, such as IL-10 and TGF-β [47]. M1 macrophages related biomarkers include TNF-α, IL-1β, IL-6, CD80, CD68, and CD86, and biomarkers of M2 macrophages include IL-10, MRC1 and Arg-1 [48]. Importantly, main phenotype of macrophages in COPD subjects is M1 [49].

S1PR1 and HDAC1 are known to be involved in regulation of macrophages, while the effects of these genes on macrophage polarization have remained an ongoing debate. In our study, we detected that downregulation of S1PR1 induced an increase in the absolute count of IL-10 positive cells, but inhibited the proliferation of IL-12 positive cells, suggesting that S1PR1 supports the M1 phenotype. Our data also showed that, S1PR1 significantly inhibited the expression of HDAC1, the number of IL-12-positive cells increased while IL-10-positive cells were impaired, thus fostering an anti-inflammatory signal. These data are also consistent with previous reports that S1P inhibited HDAC1 activity in diverse ways [50, 51]. Our results confirmed S1P/HDAC1 signaling plays an important role in regulating macrophage polarization in COPD patients. And in order to rule out the drug effects, we enrolled patients who had established diagnoses for reliable results. However, the number of patients enclosed is limited, which might compromise understandings in the results. We believe that

rophages assumed a dominant M1 phenotype. Macrophage polarization is closely related to human inflammatory response [45]. Macrophages transition between classically activated

Figure 6. S1PR1 improve the expression of HDAC1. A. The increase of S1PR1 level has influence on HAT concentration in macrophage nucleus of healthy control group (*P<0.05, n=4). B. The increase of S1PR1 level has influence on HAT concentration in macrophage cytoplasm of healthy control group (*P<0.05, n=4). C. The effect of S1PR1 overexpression on the HDAC1 concentration in the nuclei of macrophages derived from healthy controls (*P<0.05, n=4). D. The effect of S1PR1 upregulation on the HDAC1 concentration in the cytoplasm of macrophages derived from healthy controls (*P<0.05, n=4). E. The increase of S1PR1 level has influence on IL-10 concentration in the culture medium of macrophage in healthy control group (*P<0.05, n=4). F. The increase of S1PR1 level has influence on IL-12 concentration in the culture medium of macrophage in healthy control group (*P<0.05, n=4).

with the number of patients increasing, the confidence of our results will improve.

In summary, our study demonstrates a close link between macrophage polarization with COPD and its biological mechanism, which may deliver new insight into the role of S1P and HADC1. This may open new avenues for discovering treatments for COPD.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Yanwei Chen, Department of Pulmonary and Critical Care Medicine, The Second Affiliated Hospital of The Air Force Military Medical University, No. 569 Xinsi Road, Xi'an 710038, Shaanxi, PR China. E-mail: chenyw@szu.edu.cn

References

[1] Singh D, Agusti A, Anzueto A, Barnes PJ, Bourbeau J, Celli BR, Criner GJ, Frith P, Halpin

DMG, Han M, Lopez Varela MV, Martinez F, Montes de Oca M, Papi A, Pavord ID, Roche N, Sin DD, Stockley R, Vestbo J, Wedzicha JA and Vogelmeier C. Global strategy for the diagnosis, management, and prevention of chronic obstructive lung disease: the GOLD science committee report 2019. Eur Respir J 2019; 53: 1900164.

[2] Garcia-Rio F, Miravitlles M, Soriano JB, Munoz L, Duran-Tauleria E, Sanchez G, Sobradillo V and Ancochea J; EPI-SCAN Steering Committee. Systemic inflammation in chronic obstructive pulmonary disease: a population-based study. Respir Res 2010; 11: 63.

[3] Neunhauserer D, Patti A, Niederseer D, Kaiser B, Cadamuro J, Lamprecht B, Ermolao A, Studnicka M and Niebauer J. Systemic inflammation, vascular function, and endothelial progenitor cells after an exercise training intervention in COPD. Am J Med 2021; 134: e171-e180.

[4] Li D, Wu Y, Guo S, Qin J, Feng M, An Y, Zhang J, Li Y, Xiong S, Zhou H, Zeng Q, Chen L and Wen F. Circulating syndecan-1 as a novel biomarker relates to lung function, systemic inflammation, and exacerbation in COPD. Int J Chron Obstruct Pulmon Dis 2019; 14: 1933-1941.

[5] Tan DBA, Armitage J, Teo TH, Ong NE, Shin H and Moodley YP. Elevated levels of circulating exosome in COPD patients are associated with systemic inflammation. Respir Med 2017; 132: 261-264.

[6] Wu Y, Qin J, He J, Shen Y, Wang H, Li Y, Zeng Q, Dong J, An Y, Xiong S, Feng M and Wen F. Serum endostatin is a novel marker for COPD associated with lower lung function, exacerbation and systemic inflammation. Int J Chron Obstruct Pulmon Dis 2020; 15: 397-407.

[7] Barnawi J, Tran H, Jersmann H, Pitson S, Roscioli E, Hodge G, Meech R, Haberberger R and Hodge S. Potential link between the sphingosine-1-phosphate (S1P) system and defective alveolar macrophage phagocytic function in chronic obstructive pulmonary disease (COPD). PLoS One 2015; 10: e0122771.

[8] Dewhurst JA, Lea S, Hardaker E, Dungwa JV, Ravi AK and Singh D. Characterisation of lung macrophage subpopulations in COPD patients and controls. Sci Rep 2017; 7: 7143.

[9] Tetley TD. Macrophages and the pathogenesis of COPD. Chest 2002; 121 Suppl: 156S-159S.

[10] Varga T, Mounier R, Horvath A, Cuvellier S, Dumont F, Poliska S, Ardjoune H, Juban G, Nagy L and Chazaud B. Highly dynamic transcriptional signature of distinct macrophage subsets during sterile inflammation, resolution, and tissue repair. J Immunol 2016; 196: 4771-4782.

[11] Quero L, Hanser E, Manigold T, Tiaden AN and Kyburz D. TLR2 stimulation impairs anti-inflammatory activity of M2-like macrophages, generating a chimeric M1/M2 phenotype. Arthritis Res Ther 2017; 19: 245.

[12] Karunakaran I and van Echten-Deckert G. Sphingosine 1-phosphate - a double edged sword in the brain. Biochim Biophys Acta Biomembr 2017; 1859: 1573-1582.

[13] Molino S, Tate E, McKillop WM and Medin JA. Sphingolipid pathway enzymes modulate cell fate and immune responses. Immunotherapy 2017; 9: 1185-1198.

[14] Kurano M and Yatomi Y. Sphingosine 1-phosphate and atherosclerosis. J Atheroscler Thromb 2018; 25: 16-26.

[15] Mendelson K, Evans T and Hla T. Sphingosine 1-phosphate signalling. Development 2014; 141: 5-9.

[16] Peyrin-Biroulet L, Christopher R, Behan D and Lassen C. Modulation of sphingosine-1-phosphate in inflammatory bowel disease. Autoimmun Rev 2017; 16: 495-503.

[17] Maceyka M, Rohrbach T, Milstien S and Spiegel S. Role of sphingosine kinase 1 and sphingosine-1-phosphate axis in hepatocellular carcinoma. Handb Exp Pharmacol 2020; 259: 3-17.

[18] Di Pardo A, Amico E, Basit A, Armirotti A, Joshi P, Neely MD, Vuono R, Castaldo S, Digilio AF, Scalabri F, Pepe G, Elifani F, Madonna M, Jeong SK, Park BM, D'Esposito M, Bowman AB, Barker RA and Maglione V. Defective sphingosine-1-phosphate metabolism is a druggable target in Huntington's disease. Sci Rep 2017; 7: 5280.

[19] Goel K, Beatman EL, Egersdorf N, Scruggs A, Cao D, Berdyshev EV, Schweitzer KS and Petrache I. Sphingosine 1 phosphate (S1P) receptor 1 is decreased in human lung microvascular endothelial cells of smokers and mediates S1P effect on autophagy. Cells 2021; 10: 1200.

[20] Berdyshev EV, Serban KA, Schweitzer KS, Bronova IA, Mikosz A and Petrache I. Ceramide and sphingosine-1 phosphate in COPD lungs. Thorax 2021; [Epub ahead of print].

[21] Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, Luo C, Marmorstein R, Kordula T, Milstien S and Spiegel S. Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science 2009; 325: 1254-1257.

[22] Zwinderman MRH, de Weerd S and Dekker FJ. Targeting HDAC complexes in asthma and COPD. Epigenomes 2019; 3: 19.

[23] Ito K, Ito M, Elliott WM, Cosio B, Caramori G, Kon OM, Barczyk A, Hayashi S, Adcock IM, Hogg JC and Barnes PJ. Decreased histone deacetylase activity in chronic obstructive pul-

monary disease. N Engl J Med 2005; 352: 1967-1976.

[24] Chen Y, Huang P, Ai W, Li X, Guo W, Zhang J and Yang J. Histone deacetylase activity is decreased in peripheral blood monocytes in patients with COPD. J Inflamm (Lond) 2012; 9: 10.

[25] Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, macintyre N, mckay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G and Wanger J; ATS/ERS Task Force. Standardisation of spirometry. Eur Respir J 2005; 26: 319-338.

[26] Kimbrough D, Wang SH, Wright LH, Mani SK, Kasiganesan H, larue AC, Cheng Q, Nadig SN, Atkinson C and Menick DR. HDAC inhibition helps post-MI healing by modulating macrophage polarization. J Mol Cell Cardiol 2018; 119: 51-63.

[27] Barnes PJ. Role of HDAC2 in the pathophysiology of COPD. Annu Rev Physiol 2009; 71: 451-464.

[28] Yin Y, Chen S, Hakim MS, Wang W, Xu L, Dang W, Qu C, Verhaar AP, Su J, Fuhler GM, Peppelenbosch MP and Pan Q. 6-Thioguanine inhibits rotavirus replication through suppression of Rac1 GDP/GTP cycling. Antiviral Res 2018; 156: 92-101.

[29] Yin Y, Dang W, Zhou X, Xu L, Wang W, Cao W, Chen S, Su J, Cai X, Xiao S, Peppelenbosch MP and Pan Q. PI3K-Akt-mtor axis sustains rotavirus infection via the 4E-BP1 mediated autophagy pathway and represents an antiviral target. Virulence 2018; 9: 83-98.

[30] Dionne S, Duchatelier CF and Seidman EG. The influence of vitamin D on M1 and M2 macrophages in patients with Crohn's disease. Innate Immun 2017; 23: 557-565.

[31] Huang X, Li Y, Fu M and Xin HB. Polarizing macrophages in vitro. Methods Mol Biol 2018; 1784: 119-126.

[32] Mccloskey SC, Patel BD, Hinchliffe SJ, Reid ED, Wareham NJ and Lomas DA. Siblings of patients with severe chronic obstructive pulmonary disease have a significant risk of airflow obstruction. Am J Respir Crit Care Med 2001; 164: 1419-1424.

[33] Soler Artigas M, Wain LV, Repapi E, Obeidat M, Sayers I, Burton PR, Johnson T, Zhao JH, Albrecht E, Dominiczak AF, Kerr SM, Smith BH, Cadby G, Hui J, Palmer LJ, Hingorani AD, Wannamethee SG, Whincup PH, Ebrahim S, Smith GD, Barroso I, Loos RJ, Wareham NJ, Cooper C, Dennison E, Shaheen SO, Liu JZ, Marchini J; Medical Research Council National Survey of Health and Development (NSHD) Respiratory Study Team, Dahgam S, Naluai AT, Olin AC, Karrasch S, Heinrich J, Schulz H, mckeever TM, Pavord ID, Heliovaara M, Ripatti S,

Surakka I, Blakey JD, Kahonen M, Britton JR, Nyberg F, Holloway JW, Lawlor DA, Morris RW, James AL, Jackson CM, Hall IP and Tobin MD; SpiroMeta Consortium. Effect of five genetic variants associated with lung function on the risk of chronic obstructive lung disease, and their joint effects on lung function. Am J Respir Crit Care Med 2011; 184: 786-795.

[34] Repapi E, Sayers I, Wain LV, Burton PR, Johnson T, Obeidat M, Zhao JH, Ramasamy A, Zhai G, Vitart V, Huffman JE, Igl W, Albrecht E, Deloukas P, Henderson J, Granell R, McArdle WL, Rudnicka AR; Wellcome Trust Case Control Consortium, Barroso I, Loos RJ, Wareham NJ, Mustelin L, Rantanen T, Surakka I, Imboden M, Wichmann HE, Grkovic I, Jankovic S, Zgaga L, Hartikainen AL, Peltonen L, Gyllensten U, Johansson A, Zaboli G, Campbell H, Wild SH, Wilson JF, Glaser S, Homuth G, Volzke H, Mangino M, Soranzo N, Spector TD, Polasek O, Rudan I, Wright AF, Heliovaara M, Ripatti S, Pouta A, Naluai AT, Olin AC, Toren K, Cooper MN, James AL, Palmer LJ, Hingorani AD, Wannamethee SG, Whincup PH, Smith GD, Ebrahim S, mckeever TM, Pavord ID, macleod AK, Morris AD, Porteous DJ, Cooper C, Dennison E, Shaheen S, Karrasch S, Schnabel E, Schulz H, Grallert H, Bouatia-Naji N, Delplanque J, Froguel P, Blakey JD; NSHD Respiratory Study Team, Britton JR, Morris RW, Holloway JW, Lawlor DA, Hui J, Nyberg F, Jarvelin MR, Jackson C, Kahonen M, Kaprio J, Probst-Hensch NM, Koch B, Hayward C, Evans DM, Elliott P, Strachan DP, Hall IP and Tobin MD. Genome-wide association study identifies five loci associated with lung function. Nat Genet 2010; 42: 36-44.

[35] Pillai SG, Ge D, Zhu G, Kong X, Shianna KV, Need AC, Feng S, Hersh CP, Bakke P, Gulsvik A, Ruppert A, Lodrup Carlsen KC, Roses A, Anderson W, Rennard SI, Lomas DA, Silverman EK and Goldstein DB; ICGN Investigators. A genome-wide association study in chronic obstructive pulmonary disease (COPD): identification of two major susceptibility loci. PLoS Genet 2009; 5: e1000421.

[36] Cho MH, mcdonald ML, Zhou X, Mattheisen M, Castaldi PJ, Hersh CP, Demeo DL, Sylvia JS, Ziniti J, Laird NM, Lange C, Litonjua AA, Sparrow D, Casaburi R, Barr RG, Regan EA, Make BJ, Hokanson JE, Lutz S, Dudenkov TM, Farzadegan H, Hetmanski JB, Tal-Singer R, Lomas DA, Bakke P, Gulsvik A, Crapo JD, Silverman EK and Beaty TH; NETT Genetics, ICGN, ECLIPSE and COPDGene Investigators. Risk loci for chronic obstructive pulmonary disease: a genome-wide association study and meta-analysis. Lancet Respir Med 2014; 2: 214-225.

[37] Cho MH, Boutaoui N, Klanderman BJ, Sylvia JS, Ziniti JP, Hersh CP, DeMeo DL, Hunninghake

GM, Litonjua AA, Sparrow D, Lange C, Won S, Murphy JR, Beaty TH, Regan EA, Make BJ, Hokanson JE, Crapo JD, Kong X, Anderson WH, Tal-Singer R, Lomas DA, Bakke P, Gulsvik A, Pillai SG and Silverman EK. Variants in FAM13A are associated with chronic obstructive pulmonary disease. Nat Genet 2010; 42: 200-202.

[38] Hsu CW, Suk CW, Hsu YP, Chang JH, Liu CT, Huang SK and Hsu SC. Sphingosine-1-phosphate and CRP as potential combination biomarkers in discrimination of COPD with community-acquired pneumonia and acute exacerbation of COPD. Respir Res 2022; 23: 63.

[39] Rubio-Navarro A, Guerrero-Hue M, Martin-Fernandez B, Cortegano I, Olivares-Alvaro E, de Las Heras N, Alia M, de Andres B, Gaspar ML, Egido J and Moreno JA. Phenotypic characterization of macrophages from rat kidney by flow cytometry. J Vis Exp 2016; 54599.

[40] Montuschi P, Malerba M, Macis G, Mores N and Santini G. Triple inhaled therapy for chronic obstructive pulmonary disease. Drug Discov Today 2016; 21: 1820-1827.

[41] Kim SR, Song JH, Ahn JH, Lee GS, Ahn H, Yoon SI, Kang SG, Kim PH, Jeon SM, Choi EJ, Shin S, Cha Y, Cho S, Kim DE, Chang SY and Ko HJ. Antiviral and anti-inflammatory activity of budesonide against human rhinovirus infection mediated via autophagy activation. Antiviral Res 2018; 151: 87-96.

[42] Ma Y, Yuan S, Tian X, Lin S, Wei S, Hu T, Chen S, Li X, Chen S, Wu D, Wang M and Guo D. ABIN1 inhibits HDAC1 ubiquitination and protects it from both proteasome- and lysozyme-dependent degradation. J Cell Biochem 2018; 119: 3030-3043.

[43] Chaudhry BZ, Cohen JA and Conway DS. Sphingosine 1-phosphate receptor modulators for the treatment of multiple sclerosis. Neurotherapeutics 2017; 14: 859-873.

[44] Li H, Ou X, Xiong J and Wang T. HPV16E7 mediates HADC chromatin repression and down-regulation of MHC class I genes in HPV16 tumorigenic cells through interaction with an MHC class I promoter. Biochem Biophys Res Commun 2006; 349: 1315-1321.

[45] Barnawi J, Tran HB, Roscioli E, Hodge G, Jersmann H, Haberberger R and Hodge S. Prophagocytic effects of thymoquinone on cigarette smoke-exposed macrophages occur by modulation of the sphingosine-1-phosphate signalling system. COPD 2016; 13: 653-661.

[46] Yang L, Han Z, Tian L, Mai P, Zhang Y, Wang L and Li L. Sphingosine 1-phosphate receptor 2 and 3 mediate bone marrow-derived monocyte/macrophage motility in cholestatic liver injury in mice. Sci Rep 2015; 5: 13423.

[47] Li C, Xu MM, Wang K, Adler AJ, Vella AT and Zhou B. Macrophage polarization and meta-inflammation. Transl Res 2018; 191: 29-44.

[48] Martinez EC, Garg R and van Drunen Littel-van den Hurk S. Innate immune protection from pneumonia virus of mice induced by a novel immunomodulator is prolonged by dual treatment and mediated by macrophages. Antiviral Res 2019; 171: 104594.

[49] Murray PJ. Macrophage polarization. Annu Rev Physiol 2017; 79: 541-566.

[50] Shi Q, Zhao L, Xu C, Zhang L and Zhao H. High molecular weight hyaluronan suppresses macrophage M1 polarization and enhances IL-10 production in PM(2.5)-induced lung inflammation. Molecules 2019; 24: 1766.

[51] Huang H, Feng H and Zhuge D. M1 macrophage activated by notch signal pathway contributed to ventilator-induced lung injury in chronic obstructive pulmonary disease model. J Surg Res 2019; 244: 358-367.