S1P/HDAC1 Signaling Regulates Macrophage Polarization in Patients with Chronic Obstructive Pulmonary Disease

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Research

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Abstract

Throughout the world, chronic obstructive pulmonary disease (COPD) is the cause of substantial morbidity and mortality and represents a large public health burden. Previous studies reported that inflammation is intimately involved in COPD, but the underlying mechanism remains unclear. As macrophage polarization was demonstrated to regulate inflammation, we hypothesized that COPD alters macrophage polarization. To test this, we investigated in individuals with COPD the relationship between S1PR1 and HADC1 expression and macrophage inflammation via flow cytometry, qRT-PCR, gene knockdown/overexpression, and western blot analysis. We found that macrophages from COPD subjects were polarized towards an M1 phenotype with increased S1PR1 and decreased HADC1 expression. S1PR1 also inhibited HADC1 expression. Thus, S1PR1/HADC1 signaling regulates macrophage polarization. The results from this study provide insights into the pathogenesis of COPD and suggest possible therapeutic opportunities.

Introduction

Chronic obstructive pulmonary disease (COPD) results in significant morbidity and mortality [1]. COPD has been demonstrated to be the third leading cause of mortality worldwide [2]. The incidence of COPD in adults older than 40 years old is approximately 9–10% [1], and 5% of all deaths worldwide were due to this disease [3]. In China, the prevalence of COPD ranges from 1.20–8.87% [4]. Notably, the prevalence of COPD increases as smoking frequencies rise [5], while more than 90% of COPD cases stem from long-term cigarette smoking [6]. Additionally, COPD is closely associated with cardiovascular (CV) disease [7].

COPD is characterized by chronic bronchitis, destruction of small airways, and enlargement/disorganization of alveoli [8]. COPD is caused by multiple factors, such as infectious agents, including *Haemophilus influenzae*, *Moraxella catarrhine*, and *Streptococcus pneumonia* [9]. Additionally, cellular- and antibody-mediated autoimmunity is thought to cause COPD, especially the inflammatory response presenting in the lower airways [10]. Increased cell senescence, which affects lung structure, and the types and numbers of inflammatory cells and fibroblasts, may also contribute to COPD [11]. However, the pathogenesis of COPD is not fully understood.

Sphingosine 1-phosphate (S1P) plays a major role in the sphingolipid pathway [12]. Sphingolipid signaling regulates cell fate and immune responses [13]. S1P originates from ceramide inside cells, which is generated *de novo* or through the breakdown of membrane-resident sphingomyelin. S1P functions as a strong lipid mediator, regulating biological activities via its five S1P cell membrane receptors (S1P1-5) [14]. S1P has been shown to regulate cell-cell and cell-matrix interactions via secretion of its protein-coupled receptors into the extracellular environment, thus closely governing cell migration, differentiation, and survival [15]. Dysregulated S1P signaling may contribute to disease, including atherosclerosis [14], inflammatory bowel [16] and Huntington's disease [17], and hepatocellular carcinoma [18]. S1P was linked to COPD, as several sphingolipid molecules modify inflammatory lung diseases and are therapeutic targets for treating lung injury and inflammation [19].

Macrophages play an important role in the pathogenesis of COPD. As the precursor of S1P, ceramide inhibits macrophage efferocytosis in COPD [20]. M1/M2 macrophage polarization is central to several inflammatory reactions [21]. Interleukin 10 (IL-10) and IL-12 are key factors in differentiating the M1 and M2 subtypes of macrophages [22]. HDACs were also linked to macrophage activity [23, 24].

Herein, we examined expression of S1PR1 and HDAC1 in macrophages from controls and individuals with COPD, and by limiting and increasing signaling, assessed changes in cell activation.

Materials And Methods

Healthy volunteers and COPD patients

All healthy volunteers and patients enrolled in the study signed written informed consent. The study was approved by the ethics committee of Shenzhen University located in Shenzhen, China (reference NSSKH201908).

Isolation of PBMCs

PBMCs from healthy and COPD individuals were isolated using 9-mL Vacuette tubes with 3.8% sodium citrate (Greiner Bio-One, Kremsmünster, Austria). Blood samples were diluted with equal volumes of tris-buffered saline solution (TBSS, pH 7.5), inverted 10 times, and centrifuged at 1700 $\times g$ for 30 minutes at room temperature. The uppermost PBMC-containing buffy coat was removed, resuspended and transferred to 15-mL conical tubes. Cells were washed one time with cold PBS and centrifuged at 300 $\times g$ for 10 minutes. If required, red blood cell contamination was mitigated with ACK lysis buffer (Leagene, Beijing, China). The PBMCs were then washed with cold PBS and spun down. The cell viability and number were measured by a Countess Automated Cell Counter (Invitrogen, Shanghai, China) according to the manufacturer's instructions.

PBMC culture and stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF)

Isolated PBMCs were re-suspended using preheated Roswell Park Memorial Institute (RPMI) 1640 culture medium (Thermo Fisher Scientific, Shanghai, China) containing 10% fetal bovine serum (FBS, Gibco, Shanghai, China), 100 IU penicillin (Thermo Fisher Scientific, Shanghai, China), and 100 $\mu\text{g/mL}$ streptomycin (Thermo Fisher Scientific, Shanghai, China), seeded in cell culture petri dishes (Thermo Fisher Scientific, Shanghai, China), and cultured at 5% CO_2 at 37°C for 3 hours. The culture medium was refreshed to remove non-attached cells, followed by stimulation with 100 ng/mL GM-CSF. The stimulation process continued for 14 days, and the medium was refreshed every 2 days.

Macrophage induction using lipopolysaccharide (LPS)

After being stimulated by GM-CSF, cells were induced by 100 ng/mL LPS (Thermo Fisher Scientific, Shanghai, China) for 24 hours.

Flow cytometry

Flow cytometry was performed based on a standard protocol as published previously [25]. Briefly, PBMCs were dissociated with 0.25% trypsin (Sigma-Aldrich) and counted. Cells were washed twice using cold PBS, followed by adjusting the cell number to be 1×10^7 cells/mL. Cells were stained with 20 μ L of fluorochrome-conjugated antibodies (1:1000), including phycoerythrin (PE)-Cy5-conjugated human CD86 (eBioscience, San Diego, CA), fluorescein isothiocyanate (FITC)-conjugated human CD80 (eBioscience, San Diego, CA), allophycocyanin (APC)-conjugated anti-human HLA-DQ (eBioscience, San Diego, CA), and PE-conjugated anti-human CD68 (eBioscience, San Diego, CA) for 30 minutes at room temperature in the dark. Cells were washed twice using cold PBS, followed by fixation buffer (Beyotime Biotechnology, Beijing, China) for 15 minutes. Cells were then permeabilized using permeabilization buffer (Beyotime Biotechnology, Beijing, China) for 15 minutes. Cells were washed again using cold PBS and analyzed using a BD FACSCalibur benchtop flow cytometer. Incubations with matched immunoglobulin isotypes were done in parallel as controls using APC-conjugated mouse IgG1 (P3; eBioscience, San Diego, CA), Alexa Fluor 488-conjugated mouse IgG2a (eBM2a; eBioscience, San Diego, CA), PE-conjugated mouse IgG1 (P3; eBioscience, San Diego, CA), or PerCP-Cy5.5-conjugated mouse IgG1 (P3; eBioscience, San Diego, CA). The data were analyzed using FlowJo 7.6 software (Flowjo, BD, Ashland, USA).

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. Briefly, cells were lysed by Trizol (Invitrogen, Shanghai, China), followed by the addition of 200 μ L of chloroform and mixed for 30 seconds. The samples were then centrifuged at 14000 $\times$ g for 15 minutes at 4°C. An equal volume of isopropanol was added, incubated for 10 minutes, and then the samples were centrifuged at 14000 $\times$ g for 10 minutes at 4°C. Finally, 60 μ L of diethylpyrocarbonate (DEPC) water was used to resuspend the RNA. The yield and purity of RNA were determined by spectrophotometric measurements of the ratio of the UV absorbance at 260 and 280 nm by a Nanodrop 2000 (Thermo Fisher Scientific, Shanghai, China). Five hundred nanograms of RNA was used for cDNA synthesis, which was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Shanghai, China) and then stored at -20°C. The qRT-PCR amplifications were carried out using a LightCycler® 480 SYBR® Green I Mastermix (Roche, Shanghai, China) on a LightCycler 480 system. The qRT-PCR protocol is published elsewhere [26, 27]. The specificity of primers was evaluated using a dissociation curve analysis. All reactions were performed in duplicates. The comparative CT method ($2^{-\Delta\Delta CT}$)

$\Delta\Delta CT$) was used to normalize target gene expression levels according to the previous method [28]. The primers used are listed in Table 1.

Table 1

Primers used for qRT-PCR in the study.

Primers	Sequences
HDAC1-F	5' GGACTGTGTAGGCATCTTCTG
HDAC1-R	5' CAATGACAGCTCCCACAAAG
S1PR1-F	5' TAGCACATGCAGCTTCTTTC
S1PR1-R	5' TCCATGTAAACTGGGTGGTT

Plasmid transient transfection

Transient plasmid transfection was performed according to previous methods [29]. Briefly, when cell confluency researched 60% to 70%, the culture medium was removed and 1 mL of transfection medium was added. The ratio of plasmid to polyethylenimine was set at 1:3, which was then mixed, incubated at room temperature for 10 minutes, and then added into the wells of a 12-well plate containing the cells. Wells were mixed gently by swirling and then incubated for 3 hours in an incubator at 37°C with 5% CO₂. Three hours post-transfection, the old medium was removed, new medium was added, and the cells were incubated overnight in an incubator at 37°C with 5% CO₂.

Statistical analysis

All data are expressed as the mean ± SEM. GraphPad Prism 6.0 software (San Diego, CA, USA) was used to calculate statistics. A Student's *t* test was used, and *p* < 0.05 was considered significant.

Results

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

GM-CSF was used for macrophage differentiation [26]. PBMCs from healthy and COPD individuals were treated with 100 ng/mL of GM-CSF for 14 days (Figure 1). The cells were then challenged with LPS (100 ng/mL) for 24 hours to generate macrophages. Interestingly, *S1PR1* mRNA levels were significantly higher (Figure 2A, *P* < 0.001, *n* = 5), and *HDAC1* mRNA levels were lower (Figure 2B, *P* < 0.01, *n* = 5) in macrophages from COPD patients as compared to the healthy individuals. Paralleling changes in

transcript, SIRP1 protein levels were increased (Figure 2C and 2D, $P < 0.01$, $n = 4$) and HDAC1 levels decreased (Figure 2E and 2F, $P < 0.01$, $n = 5$) in macrophages from individuals with COPD compared to controls.

Macrophage polarization in the healthy and COPD groups

To characterize macrophage phenotype, ratios of antigens CD68/CD80, CD86/CD68, HLA/CD86, CD86/CD80, HLA/CD80, and HLA/CD68 were examined using flow cytometry [27]. Interestingly, a significant increase in CD68/CD80, CD86/CD68, HLA/CD86, CD86/CD80, HLA/CD80, and HLA/CD68 double-positive cells were found in macrophages from COPD individuals as compared to the healthy group (Figure 3).

Effect of S1PR1 and HDAC1 inhibition on macrophage polarization

It was not clear what role S1PR1 and HDAC1 had on macrophage polarization. Thus, macrophages were treated with shRNA to knockdown either S1PR1 (Figures 4A and 4B) and HDAC1 (Figures 4C and 4D). Knockdown of S1PR1 significantly increased IL-10 production in cells from both groups while suppression of HDCA1 decreased the production of IL-10 (Figure 4E). Conversely, knockdown of S1PR1 significantly decreased IL-12 whereas knock down of HDAC1 increased production of IL-12 in macrophages from healthy and COPD individuals (Figure 4F).

To further conform these data, macrophages from healthy controls were treated with FTY720. Similarly, limiting S1PR1 increased IL-10-positive cells, but decreased the number of IL-12-positive cells (Figure 5B), as measured by flow cytometry. Additionally, inhibition of S1PR1 export with MK571 in healthy macrophages increased the number of IL-10-positive cells while decreasing IL-12-positive cells (Figure 5C).

S1PR1 upregulated the expression of HDAC1

To investigate the relationship between S1PR1 and HDAC1, an S1PR1 over-expression plasmid was constructed and transfected into macrophages derived from the healthy subjects. Overexpression of S1PR1 significantly suppressed levels of nuclear (P < 0.05, $n = 4$, Figure 6A) and cytoplasmic histone acetyltransferase (HAT) (P < 0.05, $n = 4$, Figure 6B). Consistent with these data, overexpression of S1PR1 significantly suppressed the HDAC1 concentration in the nucleus (P < 0.05, $n = 4$, Figure 6C) and the cytoplasm (P < 0.05, $n = 4$, Figure 6D) and dramatically decreased IL-10 production (P < 0.05, $n = 4$, Figure 6E), but significantly increased IL-12 (P < 0.05, $n = 4$, Figure 6F) in macrophages from healthy individuals.

Discussion

S1P, via S1PR1, is implicated in immunological, cardiovascular, and neurological processes [30]. Both S1P and S1PR1 are ubiquitously expressed, and S1P-S1PR1 signaling has been shown to regulate immunity [30]. S1P, via S1PR1, induced proinflammatory cytokines [31]. As a deacetylating enzyme, HDAC1 is involved in innate and adaptive immunity [32]. HDAC1 influences expression of major histocompatibility complex (MHC) class I genes [33]. S1PR1 family members, including S1PR12 and S1PR13, are involved in bone marrow-derived macrophage motility [34]. S1P signaling modified inflammation and macrophage function in COPD patients [35]. Interestingly, we found that S1PR1 was highly expressed and HDAC1 was lowly expressed in COPD patients.

Macrophage polarization plays an important role in inflammation [36]. Macrophages transition between classically activated macrophages (M1) and alternatively activated macrophages (M2) [37]. M1 macrophages produce proinflammatory cytokines, including TNF- α , IL-12, and IL-1, while M2 macrophages produce anti-inflammatory cytokines, including IL-10 and TGF- β [38]. Biomarkers of M1 macrophages include TNF- α , IL-1 β , IL-6, CXCL1, CXCL2, IL-12, NOS2, CD80, CD68, and CD86, and biomarkers of M2 macrophages include IL-10, MRC1, and Arg-1 [39]. Of note, macrophages from COPD subjects assumed an M1 phenotype in [40]. We found 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 macrophages assumed a dominant M1 phenotype.

While S1PR1 and HDAC1 are known to regulate macrophages, studies regarding the effects of these genes on macrophage polarization are limited. Interestingly, a decrease in S1PR1 expression was found to increase the number of IL-10-positive cells and decrease the number of IL-12-positive cells, suggesting that S1PR1 supports an M1 phenotype. Further, S1Pr1-mediated suppression of HDAC1 increased the number of IL-12-positive cells and decreased the number of IL-10-positive cells, thus fostering an anti-inflammatory signal. These data are also consistent with other reports that S1P inhibited HDAC1 activity through several mechanisms [42] [43]. Thus, the present study systematically studied the role of macrophage polarization in COPD patients. Importantly, the model of action involving macrophage polarization was dissected. Thus, this study provides insights into the pathogenesis of COPD and useful information for the development of novel therapies for the treatment of COPD.

Declarations

Acknowledgments

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Author contributions

Y.C., W.A., Y.X., and Y.F. performed the experiments. Z.Z., Y.F., and X.W. contributed to the scientific discussion, facilities, and manuscript editing. W.A. and Y.C. conceived the project and wrote the manuscript.

Conflicts of Interest

The authors have no competing interests to declare.

Ethical approval

The study was approved by the Ethics Committee of Shenzhen Nanshan District Shekou People's Hospital, Shenzhen, China (2019-01-01).

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Figures

A**Health (GM-CSF treatment)** **B****COPD (GM-CSF treatment)**

Figure 1

Time-dependent growth of GM-CSF-stimulated macrophages derived from healthy (A) and COPD individuals (B).

Figure 2

Expression of S1PR1 and HDAC1 in healthy and COPD patients. mRNA expression of (A) S1PR1 ($P < 0.001$, $n = 4$) and (B) HDAC1 ($P < 0.01$, $n = 4$) in macrophages derived from COPD individuals as compared with healthy controls. Protein level of (C, D) S1PR1 ($P < 0.01$, $n = 4$) and (E, F) HDAC1 ($P < 0.01$, $n = 4$) in macrophages derived from COPD individuals as compared with healthy controls.

A Health

B COPD

Figure 3

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

Figure 4

Effect of the inhibition of S1PR1 and HDAC1 on macrophage polarization. (A) Representative Western blot image used to determine the effect of the shS1PR1 knockdown plasmid on macrophages derived from healthy patients. (B) Western blot of the protein levels of S1PR1 in macrophages from healthy controls transfected with the shS1PR1 plasmids ($P < 0.05$, $n = 4$). (C) Representative Western blot image used to determine the effect of the shHDAC1 knockdown plasmid on macrophages derived from healthy patients. (D) Western blot quantification of the protein level of shHDAC1 in macrophages from healthy patients transfected with the shHDAC1 plasmids ($P < 0.05$, $n = 4$). (E) The concentration of IL-10 in the culture medium of macrophages with S1PR1 and HDAC1 knockdown (* $P < 0.05$, ** $P < 0.01$, $n = 4$). (F) The concentration of IL-12 in the culture medium of macrophages with S1PR1 and HDAC1 knockdown (* $P < 0.05$, $n = 4$).

A**Control** **B****FTY720 treatment** **C****MK571 treatment** **Figure 5**

The number of IL-10- and IL-12-positive cells in the macrophages derived from healthy controls that received no treatment (A) and in the groups that were treated with S1PR1 inhibitors, FTY720 (B) and MK571 (C).

Figure 6

S1PR1 upregulated the expression of HDAC1. (A) The effect of S1PR1 overexpression on the HAT concentration in the nuclei of macrophages derived from healthy controls (* $P < 0.05$, $n = 4$). (B) The effect of S1PR1 overexpression on the HAT concentration in the cytoplasm of macrophages derived from healthy controls (* $P < 0.05$, $n = 4$). (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 overexpression on the HDAC1 concentration in the cytoplasm of macrophages derived from healthy controls (* $P < 0.05$, $n = 4$). (E) The effect of S1PR1 overexpression on the IL-10 concentration in the culture medium of macrophages derived from healthy controls (* $P < 0.05$, $n = 4$). (F) The effect of S1PR1

overexpression on the IL-12 concentration in the culture medium of macrophages derived from healthy controls (* $P < 0.05$, $n = 4$).