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ORIGINAL RESEARCH

Sputum SLC40A1 as a Novel Biomarker is Increased in Patients with Acute Exacerbation of Chronic Obstructive Pulmonary Disease

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Background: Solute carrier family 40 member 1 (SLC40A1 or Ferroportin) is a cell surface glycoprotein that participates in the efflux of cellular iron and disease pathogenesis. Induced sputum is a non-invasive method for lung sample collection. However, it remains unknown whether SLC40A1 is a potential diagnostic biomarker in induced sputum cells of patients with acute exacerbation of chronic obstructive pulmonary disease (AECOPD). We in this study aimed to investigate the expression and the anti-inflammatory role of SLC40A1 in the induced-sputum cells of AECOPD patients.

Methods: A total of 35 induced sputum samples were collected from patients with AECOPD. Flow cytometry analysis was used to determine inflammatory cell phenotypes and SLC40A1 expression. Murine RAW 264.7 cell lines were treated with cigarette smoke extract (CSE) and SLC40A1-shRNA for SLC40A1 expression in vitro. ELISA was used for measurement of pro-inflammatory cytokine expression in vitro.

Results: Flow cytometry analysis showed that sputum neutrophils were increased in AECOPD patients with 3–5 exacerbations per year compared to 1 exacerbation per year, accompanied by elevated expression of CD40 and SLC40A1 in macrophages. The lung function (FEV1%pred) was reduced with a higher COPD exacerbation rate. There was a negative correlation between the FEV1% predicted and sputum neutrophil count. Patients expressing high levels of SLC40A1 exhibited higher exacerbation rates. SLC40A1 expression levels positively correlated with sputum neutrophils and negatively correlated with predicted FEV1%. In addition, mechanical ventilation reduces sputum neutrophils and SLC40A1 expression, particularly in patients with a high exacerbation rate. Further analysis in RAW 264.7 macrophage cell lines showed that cigarette smoke extract (CSE) increased the expression of SLC40A1, TNF-α, IL-6 and IL-10 at a concentration-dependent manner. SLC40A1 knockdown increased the expression of TNF-α and IL-6 and reduced the expression of IL-10 in CSE-treated macrophages.

Conclusion: SLC40A1 in sputum macrophages is increased and closely related to AECOPD severity, it would be a potential anti-inflammatory biomarker of patients with AECOPD.

Keywords: sputum, SLC40A1, AECOPD, CSE

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a progressively worsening respiratory condition and leading cause of death worldwide. Early diagnosis and treatment are essential for effective control of acute exacerbation of COPD (AECOPD). Pathogen infection, cigarette smoke, and exposure to other pollutants are major triggers of AECOPD.[1] Currently, AECOPD severity is commonly clinically evaluated using lung function tests and pulmonary inflammation. High lung inflammation usually reflects the severity of AECOPD. Neutrophils, T lymphocytes, and eosinophils are the dominant cell components in inflamed lung tissues of AECOPD patients.[2,3] Lung tissue biopsy and bronchoalveolar

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lavage (BAL) are the commonly used samples for analysis. However, these methods are limited by invasiveness and unavailability. Thus, non-invasive induced sputum has recently been used in clinics for the collection of patient samples, owing to its safety and availability. There were very few cell components in the induced sputum of healthy subjects, most of which were lung epithelial cells and alveolar macrophages. However, the cell components in the induced sputum are significantly increased in patients with acute lung inflammation. Elevated eosinophil levels have been previously reported, accompanied by increased IL-5 and eosinophil cationic protein (ECP) levels in the sputum supernatants of asthmatic patients. Immunostaining also showed a high expression of Bcl-2 in eosinophils, contributable to eosinophilia in patients with acute asthma.[4,5] Therefore, induced sputum is an alternative method for the diagnosis of asthma and other related inflammatory lung diseases.[6]

Recently, induced sputum has been used to diagnose and evaluate COPD severity. For example, a high population of neutrophils was observed in the sputum of neutrophilic COPD patients, accompanied by high levels of pro-inflammatory cytokines and mediators, such as IL-36, IL-23,[7] 6 gene expression signature (6GS) and IL-1β.[8] However, IL-36 protein levels were decreased in the sputum of patients with eosinophilic COPD.[7] In patients with AECOPD, sputum levels of prostaglandin E2 (PGE-2), 6GS, and IL-6-dependent iron are increased, along with severe respiratory symptoms and exacerbations.[8–10] In addition, classically activated macrophages (M1 cells) exclusively express high amounts of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), and other mediators that contribute to the recruitment of neutrophils from circulation into inflamed lung tissues.[11,12] However, there is no clear evidence of the predominance of M1 cells and alternatively activated macrophages (M2 cells) in patients with COPD.[13,14] A few reports have shown reduced levels of M2 monocytes in the blood of patients with moderate and severe COPD.[15] While all M1, M2, and M0 cells were reported predominance in the sputum of patients with community-acquired pneumonia (CAP), COPD and CAP+COPD patients.[16]

To clarify the presence of macrophage subtypes and explore potential novel biomarker in sputum of AECOPD, we recruited a total of 35 patients with AECOPD and analyzed CD40+ M1 cells in the induced-sputum of these patients by flow cytometry analysis and found that CD40+ cells were significantly elevated, accompanied by increased neutrophils in the induced sputum of AECOPD with a high exacerbation rate. In addition, the expression level of SLC40A1, an anti-ferroptosis protein, was also elevated in sputum macrophages of patients with high AECOPD exacerbation rates and that was closely related to AECOPD severity. Therefore, SLC40A1 would be an additional sputum biomarker in the diagnosis and evaluation of AECOPD.

Materials and Methods

Characteristics of Patients

Thirty-five patients with AECOPD from Zhabei Center Hospital, Shanghai, China, were randomly recruited for this study from 2021 to 2023. Patient characteristics are summarized in Table 1. These patients were classified into current smokers, ex-smokers and never smokers. Previous complication with pulmonary heart disease, respiratory failure, bronchiectasis,

Table 1 Characteristics of Patients with Acute Exacerbation of Chronic Obstructive Pulmonary Disease (AECOPD)

Variable	COPD Patients
Subjects, n	35
Sex (male) (N, %)	30 (85.71)
Age (years) ($\bar{X} \pm SDs$)	70.08±1.08
Body-Mass Index	22.72±4.13
Symptoms (N, %)	
Cough + Expectoration + Polypnea	29 (82.86)
Only Polypnea	6 (17.14)

(*Continued*)

Table 1 (Continued).

Variable	COPD Patients
Smoking status (N, %)	
Current	18 (51.43)
Ever	8 (22.86)
Never	9 (25.71)
Smoking history (pack-yr)	36±21
Previous history (N, %)	
Hypertension	19 (54.29)
Diabetes	7 (20)
Arrhythmia	3 (8.57)
Cardiac Dysfunction	11 (31.42)
Coronary Artery Disease	7 (20)
Pulmonary heart disease	5 (14.29)
Respiratory failure	8 (22.86)
Bronchiectasis	1 (2.86)
Pulmonary embolism	2 (5.71)
Other complications	5 (14.29)
Treatments (N, %)	
Mono Bronchodilator	0 (0)
Dual Bronchodilator	17 (48.57)
ICS+LABA/ICS+LAMA	1 (2.85)
ICS+LABA+LAMA	14 (40)
No Inhaled Medication	3 (8.57)
FEV1 (L)	1.06±0.08
FEV1%pred	38.36±2.60
FEV1/FVC	50.53±1.71
Inflammatory cells (%) [M (P25,P75)]	
Neutrophils	54.46 (24.81,83.32)
Eosinophils	0.31 (0,1.38)
Lymphocytes	2.03 (0.76,2.90)
Macrophage	1.30 (0.69,6.04)
Airway inflammatory phenotype (N, %)	
Neutrophilic phenotype	15 (42.85)
Eosinophilic phenotype	0 (0)
Mix Granulocyte phenotype	2 (5.71)
Paucigranulocytic phenotype	18 (51.42)

Notes: Continuous data with a normal distribution are expressed as mean ±SDs, while continuous data without a normal distribution are expressed as M (P25, P75). Based on the percentages of neutrophils and eosinophils in induced sputum, the inflammation in the induced sputum sample was classified into four phenotypes, including neutrophilic phenotype, eosinophilic phenotype, mixed granulocyte phenotype and paucigranulocytic phenotype. The neutrophilic phenotype was defined as a sample with ≥ 61% neutrophils and <3% eosinophils; the eosinophilic phenotype was defined as a sample with ≥ 3% eosinophils and <61% neutrophils; the mixed phenotype was defined as a sample with ≥ 61% neutrophils and ≥ 3% eosinophils; and the paucigranulocytic phenotype was defined as a sample with < 60% neutrophils and < 3% eosinophils.

Abbreviations: N, number; mean±SD, mean±standard deviation; M, median; FVC, forced vital capacity; FEV1, forced expiratory volume in one second; The body-mass index is the weight in kilograms divided by the square of the height in meters. ICS, inhaled corticosteroids; LABA, long-acting β-agonist; LAMA, long-acting muscarinic antagonist.

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pulmonary embolism was indicated in Table 1. There was no asthma complication in the recruited patients. The inclusion criteria were as follows: 1) diagnosis by a pulmonary physician according to GOLD guidelines; 2) qualified induced sputum and > 0.3×10^6 total cell counts in the induced sputum; and 3) acute exacerbation when patients were hospitalized at least once a year. Exclusion criteria included any of the following: 1) induced sputum failure (defined as invisible phlegm cell mass by the naked eye), and 2) invalid sputum samples (squamous epithelial cells≥20%). The study was approved by the Medical Ethics Committee of the Zhabei Center Hospital, Shanghai, China, and was conducted in accordance with the principles of the Declaration of Helsinki. Signed informed consent to the inclusion criteria was obtained from all participants before the study was initiated.

Sputum Induction

Patients with AECOPD inhaled 200 μg salbutamol, followed by inhalation of 4.5% NaCl for 5 min. The previous process was repeated three times if there was insufficient induced sputum. All sputum plugs with visible solidity were immediately diluted 4 times with a mixture of 0.1% dithiothreitol (DTT) and phosphate- buffered saline (PBS), then shaken for 10 min at room temperature. After filtration through two layers of sterile gauze, the induced sputum was centrifuged at 1500 rpm for 10 min. The cells were immediately collected for analysis or frozen in freezing medium for future analysis.

Noninvasive Mechanical Ventilation

According to the guidelines,[17] patients received noninvasive ventilation (NIV) through a total full-face or oro-nasal mask and were monitored with continuous SpO2, electrocardiography, and noninvasive blood pressure measurements. The inspired oxygen fraction (FiO2) was set to maintain the peripheral oxygen saturation (SpO2) between 88% and 92%. The ventilator was set in Pressure Support Ventilation (PSV) mode, with a positive end-expiratory pressure (PEEP) titrated between 3 and 5 cm H2O. The inspiratory pressure was titrated to achieve a measured or estimated expiratory tidal volume of 6–8 mL kg−1 of ideal body weight.[18]

Spirometry

Lung function was measured by spirometry analysis, which was performed by a trained respiratory technician according to American Thoracic Society recommendations.[19] Chronic airflow limitation (CAL) or FEV1% is defined as the fixed ratio of forced expiratory volume in 1 s (FEV1) to forced vital capacity (FVC) after bronchodilation. FEV1%pred was defined as the percentage of patient airflow relative to the average airflow of other people of the same age, sex, and body type. The values of FEV1% predicted at ≥70%, 60–69%, 50–59%, 35–49% and <35% were defined as mild, moderate, moderate-to-severe, severe, and extremely severe COPD, respectively.

Flow Cytometry Assay

$0.1–0.5\times10^6$ single cell suspension was incubated with an antibody cocktail containing PE-Cy7-conjugated anti-Ly6G, AF-790-conjugated anti-Ferroportin (SLC40A1), FITC-conjugated anti-CD11b and PE-conjugated anti-CD40 antibodies (BioLegend. San Diego, CA, USA) for 30 minutes in the dark. Fluorescence minus one (FMO) and isotype IgG staining were used as the controls. Stained cells were analyzed using a BD FACSAria™ II flow cytometer. All data were analyzed using FlowJo software, version 8.8.4 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Cell Culture and Treatment

Murine RAW 264.7 cell lines (ATCC, Manassas, VA) were treated with 4% cigarette smoke extract (CSE) or infected with SLC40A1-shRNA/GFP-encoding lentiviral vector at a multiplicity of infection (MOI) of 20 (sh-SLC40A1 sequence: 5′-GUCUGUUUCUCCAUUUGAA-3). Cells treated with scrambled shRNA/GFP vectors were used as controls (sh-Scramble RNA sequence: 5′-GGCACAAGCUGGAGUACAA-3′) (Guangzhou RiboBio, China). The transfected cells were selected with 4 μg/mL puromycin for 3 weeks to obtain SLC40A1 knock-down cell stables.

ELISA Assay

The concentrations of IL-6, IL-10, and TNF-α in the cell lysates were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The data were normalized to the total protein content of the cell lysates.

Statistical Analysis

The results are presented as the mean ± standard error of each group. All data were statistically analyzed using the GraphPad Prism 7 software. Student's *t* test was performed for comparisons between two groups, and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was performed for over two groups. A value of $p<0.05$ was considered statistically significant different.

Results

The Sputum Neutrophils and CD40+ Macrophages Were Increased in AECOPD Patients

All collected sputum samples were processed for flow cytometry after staining with antibody cocktails. CD11b+Ly6G+ cells were identified as neutrophils and CD11b+Ly6G- cells were identified as macrophages. The expression of CD40 and SLC40A1 was further analyzed in gated macrophages (Figure 1A). The cell samples were divided into three groups: AE1, AE2, and AE3. AE1, AE2, and AE3 were classified as 1, 2, and 3–5 exacerbation rates/year, respectively. As shown in Figure 1B and C, there was a gradual increase in the percentage of neutrophils (32.78±2.81 vs 48.65±5.64%) and CD40+ M1 cells (22.14±1.92 vs 31.46±3.12%) in the AE3 group compared to that in the AE1 group. In addition, all samples from the AECOPD group exhibited a high population of neutrophils and CD40+ M1 cells compared to those in the stable COPD group (data not shown).

Higher Sputum Neutrophils are Associated to Lower Lung Functionality

Of the 35 recruited AECOPD patients, 20 had one exacerbation (AE1 group), 9 had two exacerbations (AE2 group), and 6 had 3–5 exacerbations per year (AE3 group). Lung function, including FEV1%pred and FEV1/FVC, was gradually and significantly reduced in patients with high exacerbation rates compared with those with low exacerbation rates (45.79 ±3.07% vs 27.46±5.66%; 55.62±1.69% vs 42.55±3.43%) (Figure 2A). There was a significantly negative correlation between FEV1%pred and sputum neutrophils (R^2=0.41, p<0.0001), indicating a significant involvement of neutrophils in AECOPD severity (Figure 2B).

Patients with High Exacerbation Rate Had Elevated SLC40A1 Expression in Sputum Macrophages

Further analysis indicated that sputum macrophages from patients with high exacerbations (AE3) expressed higher SLC40A1 than those from patients with lower exacerbations (AE1) (9.83±1.09% vs 14.83±1.44%, Figure 3A). When patients were divided into SLC40A1(low) and SLC40A1(high) expression groups at a cut-off value of 11% SCL40A1+ macrophages, a higher exacerbation rate was observed in patients of SLC40A1(high) group than in those of SLC40A1 (low) group (0.82±0.14 vs 1.73±0.21; Figure 3B). Further analysis showed that there was a significantly positive correlation between SLC40A1 expression and sputum neutrophils (R^2=0.48, p<0.0001, Figure 3C), but that was negatively correlated to predicted FEV1% (R^2=0.41, p<0.0001, Figure 4). The results indicated that sputum macrophage-expressed SLC40A1 would be a predictor of lung inflammation and AECOPD severity, and that can be used as a potential biomarker for the diagnosis and evaluation of AECOPD.

Mechanical Ventilation Reduced Sputum Neutrophils and the Expression of SLC40A1

Non-invasive mechanical ventilation is commonly used and has proven effective in reducing the incidence of complications and improving respiratory function in patients with AECOPD.[20] Further analysis in patients with mechanical ventilation showed that mechanical ventilation significantly reduced sputum neutrophils, particularly among patients in

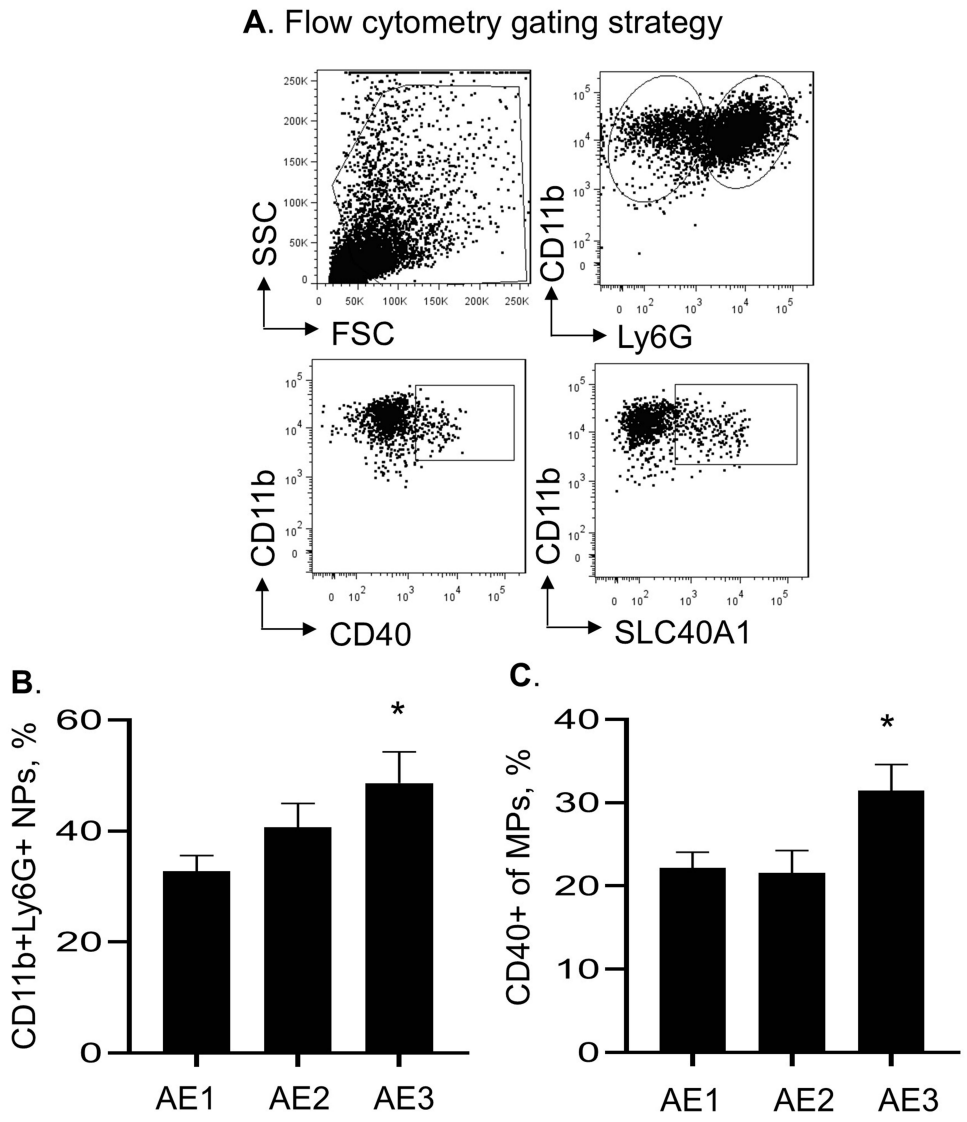

Figure 1 Neutrophils and CD40+ M1 macrophages were increased in sputum of AECOPD patients. (**A**) Gating strategy of flow cytometry analysis. CD11b+Ly6G+ cells present sputum neutrophils (NPs); CD11b+Ly6G- cells presents sputum macrophages (MPs). CD40+ M1 cells and SLC40A1+ cells were gated on sputum macrophages. (**B** and **C**) Quantitative analysis of neutrophils and CD40+ M1 cells. Data was presented as mean ± standard error. AE1 (n=20), AE2 (n=9) and AE3 (n=6) present 1, 2, and 3–5 exacerbations a year, respectively. *p<0.05, vs AE1 group.

the AE3 group, compared to those unventilated patients (50.87±5.85% vs 23.44±3.89%; Figure 5A). Supporting these results, mechanical ventilation reduced the expression of SLC40A1 in sputum macrophages, as compared to those unventilated patients (19.17±0.84% vs 14.50±1.13%; Figure 5B). The results further indicated SLC40A1 as a potential indicator of AECOPD severity.

Cigarette Smoke Extract (CSE) Up-Regulated the Expression of SLC40A1 and Pro-Inflammatory Cytokines in Macrophages

To further investigate the role of CSE in regulation of SLC40A1 expression in vitro, we treated RAW 264.7 macrophage cell lines with different concentration CSE for 24 hrs and the expression of SLC40A1 was analyzed by flow cytometry analysis. The results showed that CSE effectively upregulated the expression of SLC40A1 in a concentration-dependent manner (Figure 6A and B), accompanied by increased expression levels of pro-inflammatory cytokines (TNF-α and IL-6) and the anti-inflammatory cytokine IL-10 (Figure 6C and D). However, the upregulated IL-10 expression reached

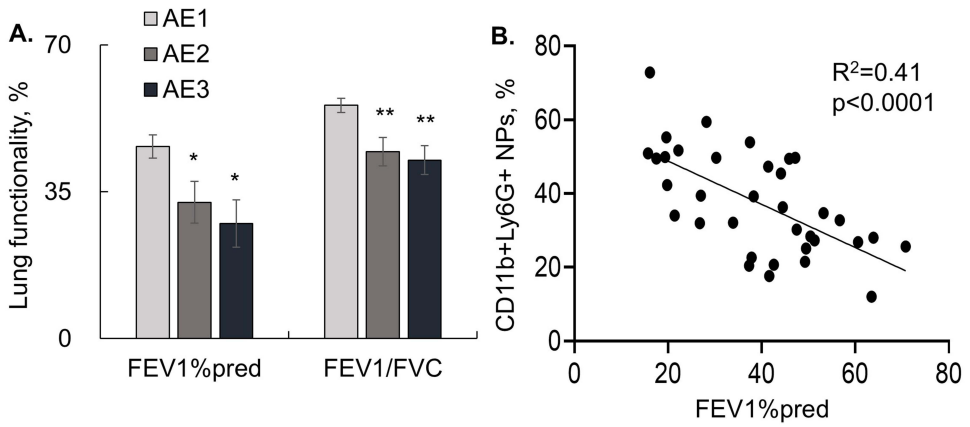

Figure 2 More sputum neutrophils are associated with lower lung functionality. (**A**) Lung function was tested by spirometry among AE1, AE2 and AE3 groups of patients in. Mean ± standard error, *p<0.05, **p<0.01 vs AE1 group. (**B**) Correlation analysis between FEV1%pred and the percentage of sputum neutrophils. Each dot presents individual patient sample.

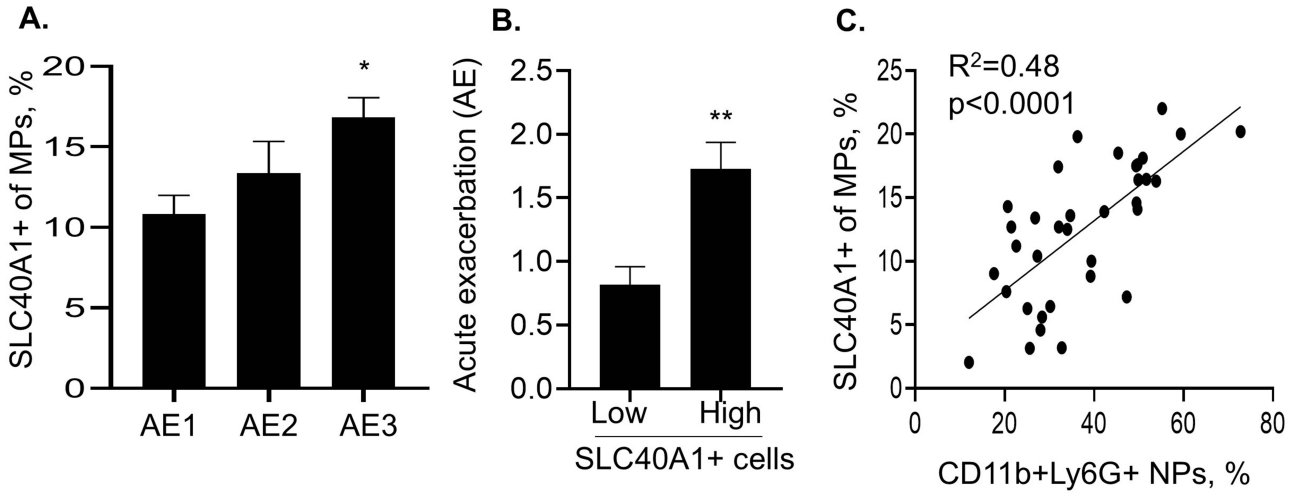

Figure 3 Patients with high exacerbation rate had more expression of SLC40A1 in sputum macrophages. (**A**) Quantitative analysis of sputum SLC40A1+ macrophages after flow cytometry analysis, *p<0.05, vs AE1 group. (**B**) The cut-off value of SCL40A1 at 11% was used to divide into SLC40A1(low) and SLC40A1(high) groups, which are ranging from 1.93% to 10.7% in SLC40A1(low) and from 11.2% to 22% in SLC40A1(high) groups. Data was presented as mean exacerbation rate a year (AE) ± standard error, **p<0.01, vs SLC40A1(low) group. (**C**) Correlation analysis between SLC40A1+ macrophages and sputum neutrophils. Each dot presents individual patient sample.

a plateau at low CSE concentrations and was then attenuated to levels comparable to those of untreated cells. Therefore, CSE increases the expression of SLC40A1 and pro-inflammatory cytokines in macrophages.

Knockdown of SLC40A1 Expression Increased the Expression of Pro-Inflammatory Cytokines in CSE-Treated Macrophages

To further explore the biological function of SLC40A1 in macrophages, we knock-down the expression of SLC40A1 in macrophages by infection with lentiviral vectors encoding shRNA-SLC40A1. shRNA-NC was used as a control. The two vectors contained inserts that were fused to the GFP reporter gene. After selection with puromycin for 3 weeks, a 42% reduction in SLC40A1 expression was observed compared to scramble shRNA-treated cells, as analyzed by flow cytometry (Figure 7A and B). Macrophages treated with 4% CSE had increased expression of IL-6, TNF-α and IL-10, compared to untreated cells. SLC40A1 knockdown further increased the expression of IL-6 (2.26±0.25 vs 4.11±0.27 ng/mg protein) and TNF-α (254.17±66.9 vs 404.04±102.43 pg/mg protein) in CSE-treated cells. However, IL-10 was downregulated after SLC40A1 knockdown in the macrophages (1936.57±706.50 vs 417.31±269.30 pg/mg protein;

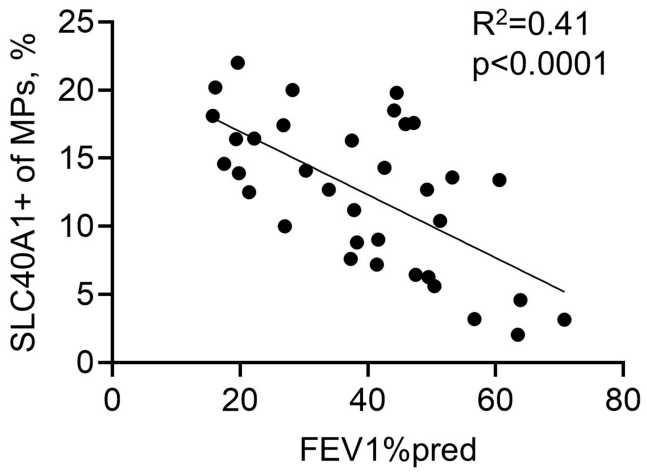

Figure 4 Correlation analysis between sputum SLC40A1+ macrophages and lung functionality (FEV1%pred). Each dot presents individual patient sample.

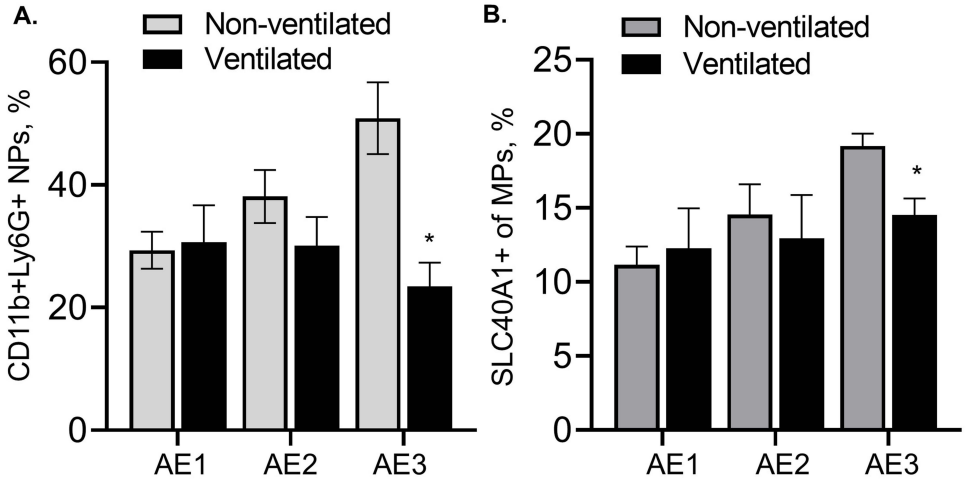

Figure 5 Mechanical ventilation reduced sputum neutrophils and the expression of SLC40A1. Patients were divided into groups treated with or without mechanical ventilation. The population of CD11b+Ly6G+ neutrophils (**A**) and SLC40A1+ (**B**) macrophages were analyzed by flow cytometry analysis in patients with indicated exacerbation rates. SLC40A1+ cells were gated on macrophages. Data was presented as mean±standard error, n=3-17/group, *p<0.05, vs None-ventilated group.

Figure 7C–E). These results indicated an anti-inflammatory role of SLC40A1 in macrophages, and SLC40A1 could protect patients from the progression of AECOPD.

Discussion

Induced sputum is widely used in the diagnosis and evaluation of pulmonary diseases such as chronic obstructive pulmonary disease (COPD), asthma, and acute respiratory distress syndrome (ARDS). Sample quality control is an important step in induced sputum-related research and clinical practice. We usually discarded samples with over 20% epithelial cells, cell amounts lower than 0.3×10^6 cells, and that contained too many debris. A total of 35 samples met these criteria and were included in this study. All samples were collected from hospitalized patients with AECOPD. Flow cytometry was used to analyze cell components and gene expression in the cell subsets. To our knowledge, this is the first report using flow cytometry analysis to show that neutrophils are markedly increased in the induced sputum of patients with frequent exacerbations of AECOPD. These results are consistent with a previous report, in which the ratio of neutrophils to lymphocytes (NLR) in serum was significantly increased and associated with 9% death in AECOPD patients at 28 days.[3] It is known that neutrophils significantly contribute to the progression of AECOPD, because they

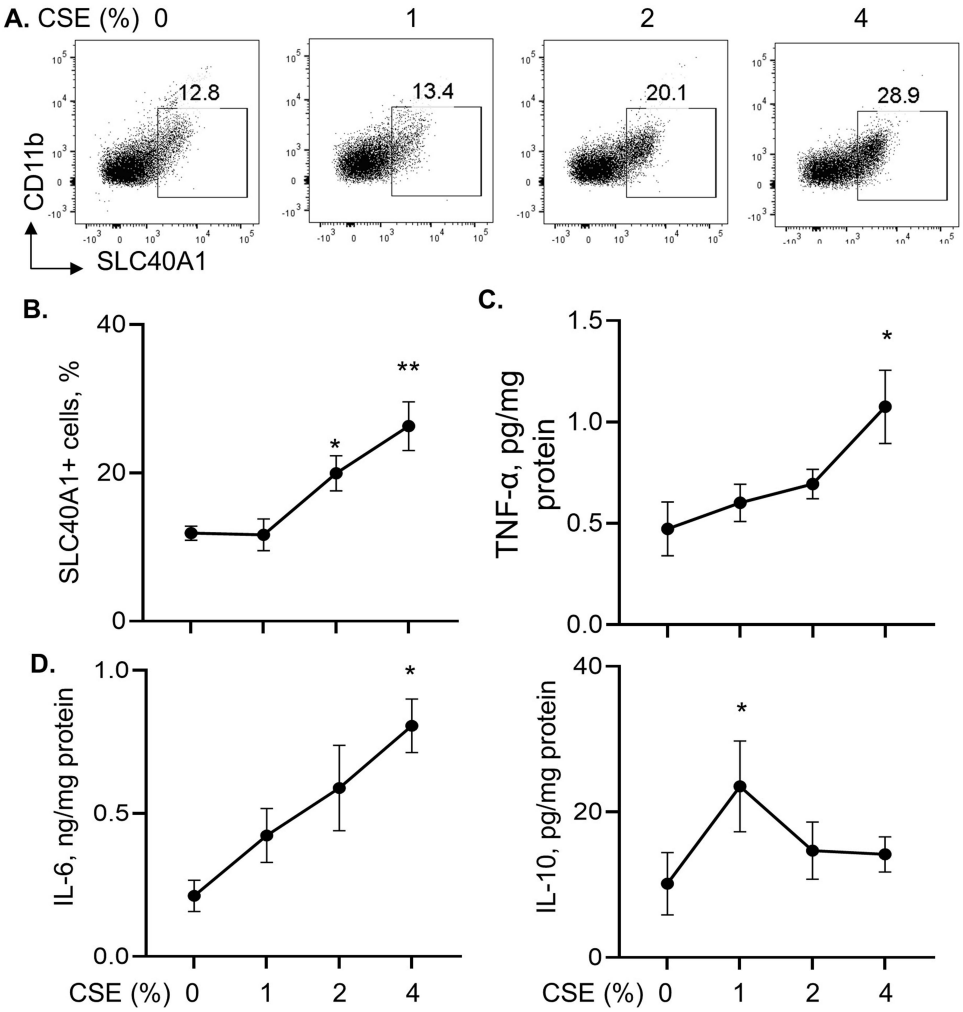

Figure 6 Cigarette smoke extract (CSE) up-regulated the expression of SLC40A1, TNF-α, IL-6 and IL-10 in macrophages at a concentration-dependent manner. (**A**) Flow cytometry analysis for SLC40A1 expression in the CSE-treated macrophage. RAW 264.7 cell lines were treated with indicated concentration of CSE for 24 hrs or untreated. Representative dot plots. (**B**) Quantitative analysis for SLC40A1+ cells after flow cytometry. (**C** and **D**) ELISA analysis for the expression of TNF-α, IL-6 and IL-10 in CSE-treated cell lysates. Data was presented as ratio of cytokines to total cell protein lysates. Mean ± standard error for each group. n=3, *p<0.05, **p<0.01 vs untreated group.

release large amounts of potent pro-inflammatory cytokines, mediators, and proteases such as IL-6, IL-1β, TNF-α, IL-8, metalloproteinase-9 (MMP-9) and neutrophil elastase after stimulation.[2,21,22] The destructive effects of neutrophils on the lungs of AECOPD patients are reflected by their lower lung functionality. Thus, flow cytometric analysis of sputum neutrophils is a simple and rapid method for the diagnosis and evaluation of AECOPD severity.

In addition, we observed high expression of CD40+ M1 cells in the sputum of patients with a high exacerbation rate. M1 cells contain high amounts of intracellular Fe^{2+} and express high amounts of transferrin receptor CD71, ferrireductase Steap3, pro-inflammatory cytokines, chemokines, and mediators. These mediators significantly contribute to the chemotaxis of neutrophils and lymphocytes into inflamed lung tissues, exaggerating immune responses, and destroying lung tissues in AECOPD.[23–25] Thus, this study confirmed that sputum macrophage activation was involved in AECOPD exacerbation.

The novelty of this study was that we observed a high expression of SLC40A1 in sputum macrophages of AECOPD patients with high exacerbations, as analyzed by flow cytometry. To our knowledge, it is the first time to analyze protein expression in sputum cells of AECOPD by flow cytometry. The method is easily integrated into current diagnostic protocols and applicable to clinical use, compared to other previously used methods, such as qRT-PCR, ELISA and immunostaining in sputum cells,[5,7,8] because flow cytometry method is simple and fast, not significantly influenced by variability of sputum volume and collected sputum cell number. By flow cytometry analysis, we also observed moderate

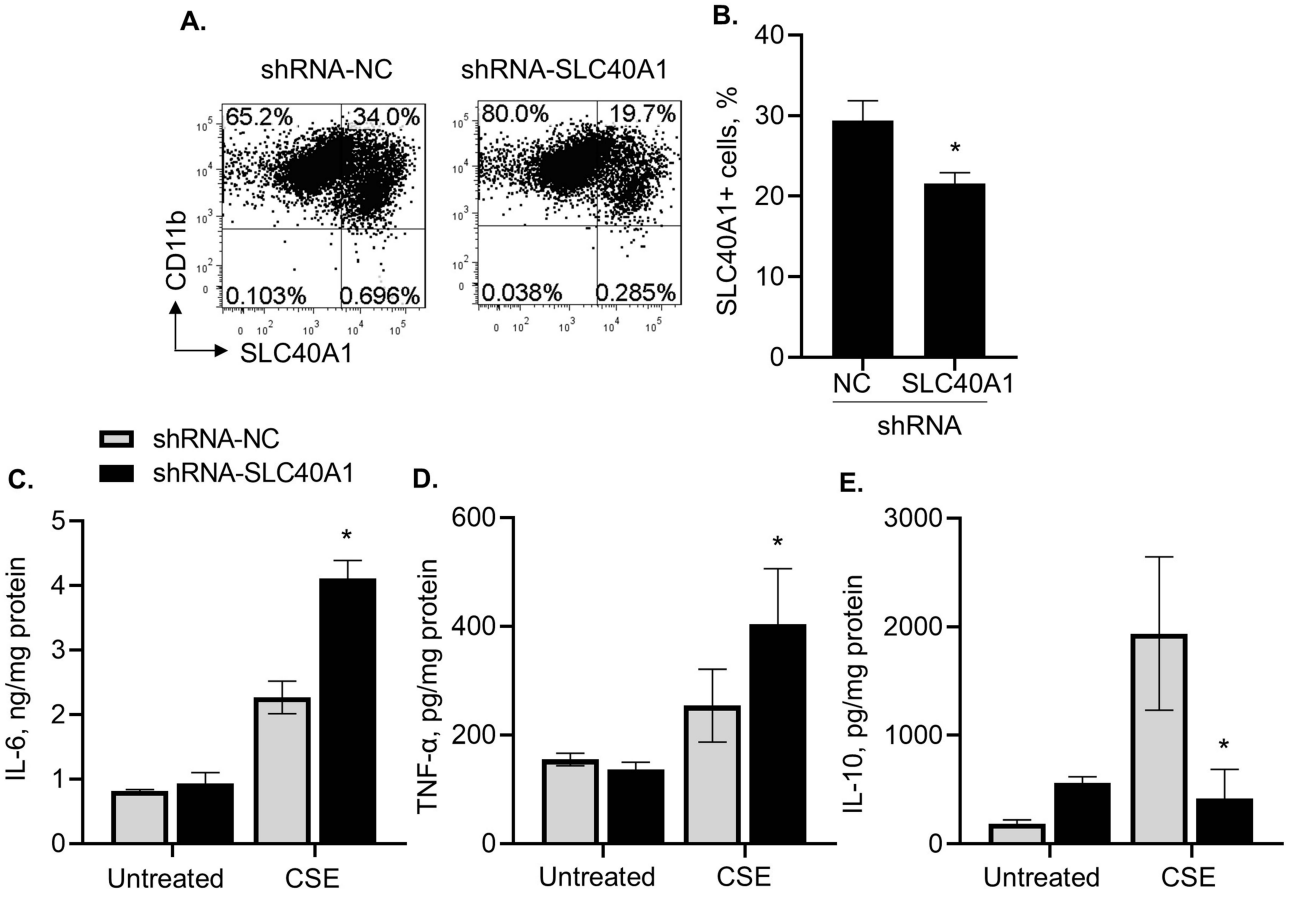

Figure 7 Knockdown of SLC40A1 expression increased the expression of TNF-α, IL-6, and reduced the expression of IL-10 in CSE-treated macrophages. (**A**) Flow cytometry analysis for SLC40A1 expression in RAW 264.7 cells that was transfected with lentiviral vector encoding shRNA-SLC40A1. shRNA-NC were control cells transfected with empty lentiviral vector encoding GFP reporter gene alone. (**A**) Representative dot plots. (**B**) Quantitative analysis of SLC40A1 expression in RAW 264.7 cells. (**C**–**E**) ELISA analysis for the expression of IL-6, TNF-α and IL-10 in the CSE-treated cell lysates. Data was presented as a ratio of cytokines to total cell protein lysates. Mean ± standard error for each group. n=3, *p<0.05 vs untreated or NC group.

positive correlation of SLC40A1 expression to lung inflammation, but negative correlation to lung functionality. Therefore, we speculate that SLC40A1 is a potential biomarker in the diagnosis and evaluation of AECOPD severity. It was previously reported that high expression of SLC40A1 was also increased in pulmonary macrophages of patients with ARDS and bacteria-infected mice. The increased SLC40A1 may exert pathological function through Yes-associated protein signaling, because degradation of SLC40A1 by C-Hep, the self-assembled N-terminally cholesterylated mini-hepcidin protected mice from bacteria-induced lung injury.[26] However, SLC40A1 may play a protective role in asthma, because recent report showed that low levels of SLC40A1 in BAL of childhood allergic asthma patients are associated with the development of type 2 airway inflammatory markers.[27] The underlying mechanisms why SLC40A1 exerts distinct roles in various inflammatory lung diseases are not well defined so far, that should be clarified in the future.

Pulmonary iron levels are increased in COPD and emphysema and associated with increased susceptibility to infections and disease severity.[28,29] SLC40A1 is mainly expressed on the surface of lung epithelial cells, myeloid cells, and cancer cells,[24,30,31] and is responsible for the efflux of intracellular Fe^{2+} and maintaining optimal mitochondrial metabolism by interacting with Steap4.[32,33] However, this activity is blocked by intracellular hepcidin.[34] It is essential to maintain a balanced level of intracellular Fe^{2+} and SLC40A1 expression under physiological condition, because low levels of SLC40A1 or deficiency causes accumulation of toxic intracellular Fe^{2+} and cell ferroptosis.[35] Low expression of SLC40A1 has been reported to contribute to the progression of breast cancer owing to the increased demand for intracellular iron by cancer cells.[30] In contrast, high levels of SLC40A1 can cause excessive export of toxic Fe^{2+} across the cell membrane and reduce intracellular Fe^{2+} levels, ultimately reducing hemoglobin synthesis and development of

fatal heart failure, due to induced mitochondrial dysfunction, oxidative stress and apoptosis.[32,36] However, its role in AECOPD and other inflammatory lung diseases is not well-defined. A previous report showed that high hemosiderin levels were observed in sputum macrophages of AECOPD patients, implying a possible role of SLC40A1 in cell ferroptosis of AECOPD.[37] TNF-α, IL-6, IL-1beta, IL-8, IL-17A, granulocyte colony stimulating factor (G-CSF), interferon gamma (IFN-γ), protein (IP-)10, macrophage inflammatory protein (MIP)-1α, MIP-1β and TNF-α levels were elevated in the sputum of COPD patients and associated with high neutrophil levels in COPD.[38] Thus, we speculate that neutrophils-derived pro-inflammatory cytokines upregulate the expression of SLC40A1 and subsequently exert anti-ferroptosis and anti-inflammatory roles through a feedback loop in AECOPD. Consistent with this concept, our further in vitro studies have revealed that CSE upregulates SLC40A1, TNF-α, and IL-6 in macrophages, indicating the role of smoke exposure in upregulation of SLC40A1 and pro-inflammatory cytokines. In contrast, knockdown of SLC40A1 expression significantly increased the expression of TNF-α and IL-6; but reduced the expression of IL-10 in CSE-treated macrophages, indicating the anti-inflammatory role of SLC40A1. The results were consistent with previous report, in which SLC40A1 was upregulated by tuberculosis and IFN-γ in RAW264.7 cell lines and alveolar macrophages.[39,40] Nuclear factor erythroid 2-related factor 2 (NRF2), an anti-oxidant transcription factor is induced by ferroptosis inhibitors Ferr-1 and Lip-1, providing protection of lung A549 cells from Paraquat-induced cell injury through upregulation of SLC40A1 expression.[41] The results imply possible involvement of NRF2 signaling in SLC40A1 upregulation in AECOPD patients. However, a suppressive role of NRF2 signaling on SLC40A1 expression was also reported in cisplatin-resistant ovarian cancer cells.[42] Though we observed the up-regulated SLC40A1 expression in CSE and pro-inflammatory cytokines-treated macrophages, an opposite effect was also previously reported in the myocardium, in which LPS can induce iron deposition and reduce the expression of SLC40A1 through up-regulation of TNF-α, IL-1β and IL-6.[43] Thus, regulation of SLC40A1 expression should be further clarified under different microenvironment and cell types in the future. Based on well-documented protective role of SLC40A1 in reducing iron overload and lipid peroxidation,[41] we speculated that COPD patients have a high accumulation of iron and oxidative stress, which stimulates SLC40A1 expression to provide a protective feedback loop. However, it is not well defined so far how SLC40A1 mechanistically interacts with inflammatory pathways or impacts disease progression. More study would be required to further investigate the interaction between SLC40A1 and inflammatory signaling pathways in macrophages of AECOPD.

It is noted that study was limited by the relatively small sample size or variability in sample collection techniques during different collection time, and that lead to lower statistical power and moderate correlation between SLC40A1 expression in sputum macrophages and neutrophils, as well as lung functionality (Figures 3C and 4). In addition, patients with stable COPD, current smoker, ex-smoker and never smoker without AECOPD were not included in this study, due to difficulties in obtaining enough sputum cells. These limitations warrant us to improve sample collection techniques, enlarge sputum sample size and recruit more groups of patients in the future, towards further exploring influence of potential confounders in regulation of SLC40A1 expression and lung inflammation in larger cohorts. Overall, this study opens avenues for further investigation of SLC40A1 into diagnostic and therapeutic approaches.

Ethics Statement

Human samples were collected following written informed consent from all participants according to the Declaration of Helsinki and were granted by the Ethics Committee of Zhabei Central Hospital, Jing'an District.

Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all of these areas. All authors took part in drafting, revising or critically reviewing the article, gave final approval of the version to be published, and agree on the journal to which the article has been submitted and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this study.

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