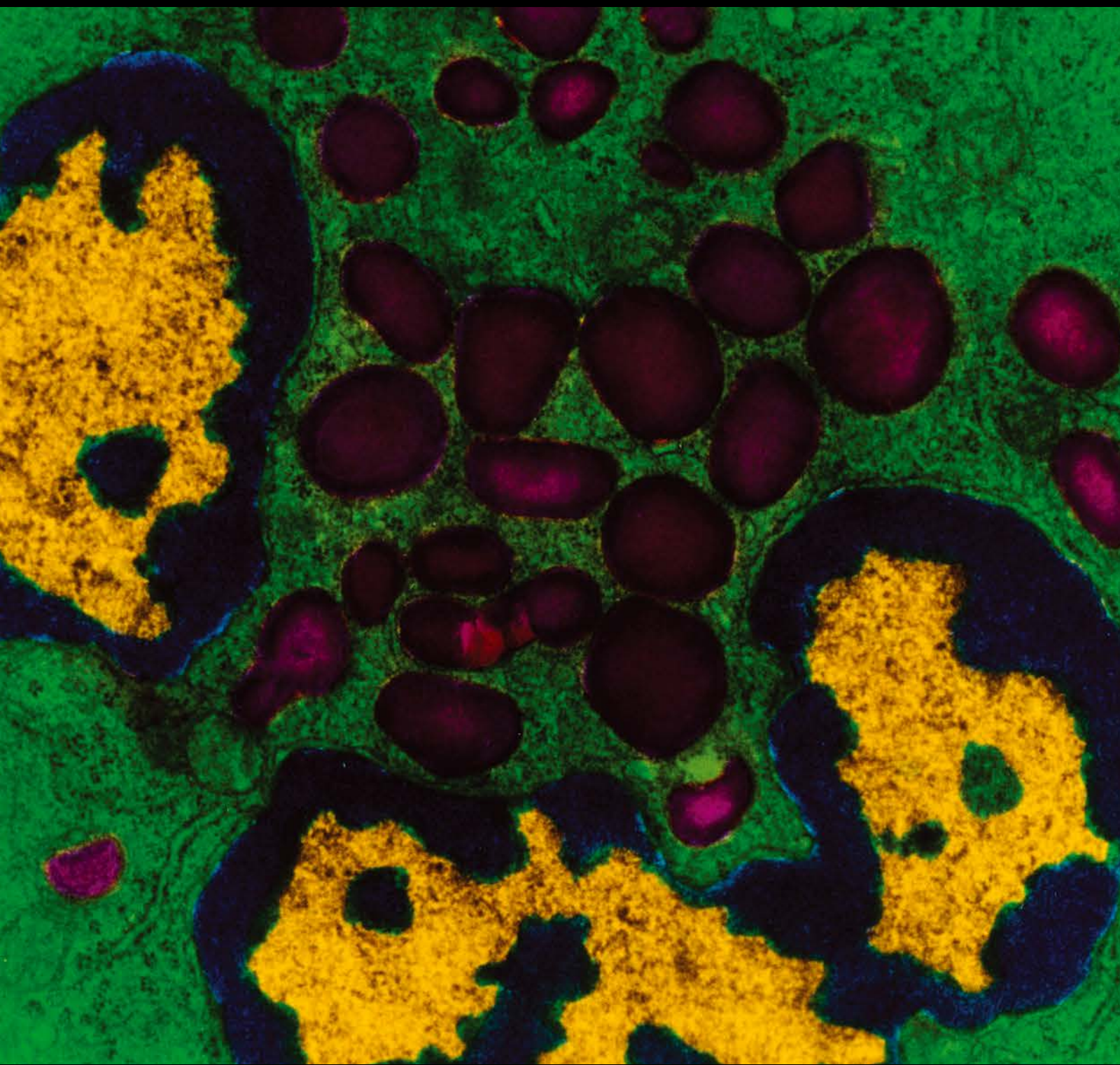
Mediators of Inflammation

Inflammation to Pulmonary Diseases

Guest Editors: Kang-Yun Lee, Kazuhiro Ito, and Kittipong Maneechotesuwan



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Editorial Board

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Editorial

Inflammation to Pulmonary Diseases

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Inflammation, which is induced and perpetuated by microorganism pathogens or from the damage or death of host cells, is an essential component of various common respiratory diseases, such as chronic obstructive pulmonary disease (COPD), asthma, bronchiectasis, and acute respiratory distress syndrome (ARDS), as well as of less common ones, for example, sarcoidosis. Although different diseases express distinct inflammatory responses, there are some shared common features. For example, the Th2-related eosinophilic inflammation, which is typically present in asthma, is also characteristic of eosinophilic phenotype of COPD. On the other hand, the innate neutrophilic inflammation shared by a number of diseases, including COPD and ARDS, could be demonstrated in some patients with severe asthma. In this special issue, common inflammation was dissected in diverse ways by the authors to better understand the related respiratory diseases, from the clinical to the underlying mechanisms, pointing to future therapeutic prospect.

Innate immunity plays a primary role in host defense against microorganism pathogens and in response to the damage or death of host cells; however, when dysregulated, instead, it changes to an essential component of various respiratory diseases. In this issue, neutrophilic inflammation was demonstrated to be involved in two clinical conditions poorly understood. F. Tang et al. investigated the neutrophilic inflammation in patients with suboptimally controlled asthma and observed a defective innate immunity, which might be linked to pathogen-induced exacerbations. F. L. Dente et al. suggested sputum neutrophilic inflammation as a good biomarker for disease severity of stable noncystic fibrosis bronchiectasis by careful investigation of correlation with clinical parameters in those subjects. K. Baines et al. unearth the role of the innate immune antimicrobial peptide β-defensin-1 in COPD and severe asthma, which is involved in persistent inflammation in those conditions and they also suggested it as a potential biomarker in sputum and a therapeutic target. Using similar approach, A. K. Barton et al. correlated BAL MMPs/TIMPs with clinical and cytological findings in different equine chronic pneumopathies and suggested that their ratios can be a good biomarker for disease severity and used for identifying subclinical cases.

A number of the articles addressed the regulatory mechanisms or novel therapies for ARDS, a disease with overwhelming nonspecific inflammation evoked by variable etiology. L. Ma et al. tested the protective roles of 3,5,4′-tri-O-acetylresveratrol (AC-Rsv), a prodrug of resveratrol, in LPS-induced ARDS, focusing on the modulation of SIRT1 and the mitogen-activated protein kinase (MAPK) pathway. In a review article, Z. Xu et al. on the other hand gave attention to the dark side of histones, the extracellular histones, acting as new members of damage-associated molecular pattern (DAMP) molecules. C. F. Gonçalves-de-Albuquerque et al. extensively reviewed the oleic acid-induced lung injury and ARDS. In addition to discussing the featured pathophysiological changes and the underlying pathogenesis, they also shed light on a link between lipid metabolism and inflammatory diseases. Finally, W.-C. Chou et al. reported a therapeutic role of caffeine in mitigating acute lung injury induced by ischemia-reperfusion of lower limbs.

To combat the pathogens evading the innate response, the immune system mounts adaptive immunity. The immune response to tuberculosis is such a prototype, for example, Th1 immune response. However, in the absence of known etiology, granulomatous inflammation characterized with infiltration of activated Th1/Th17 lymphocytes as well as macrophages features the pathological changes of pulmonary sarcoidosis. By using correlation network analysis into BAL cells in sarcoidosis, T. Dyskova et al. addressed the contribution of both microRNAs and the Th1-transcription factor T-bet to the regulation of cytokine/chemokine-receptor network, which drives the inflammatory granulomatous disease.

Thus, inflammatory pulmonary diseases, as shown in this special issue, usually recapture the inflammation evoked by the immune system to the invading pathogens or other environmental stress. Thus, one mechanism might underlie variable diseases, directing to a common therapy.

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Research Article

Neutrophilic Bronchial Inflammation Correlates with Clinical and Functional Findings in Patients with Noncystic Fibrosis Bronchiectasis

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Background. Neutrophilic bronchial inflammation is a main feature of bronchiectasis, but not much is known about its relationship with other disease features. *Aim*. To compare airway inflammatory markers with clinical and functional findings in subjects with stable noncystic fibrosis bronchiectasis (NCFB). *Methods*. 152 NFCB patients (62.6 years; females: 57.2%) underwent clinical and functional cross-sectional evaluation, including microbiologic and inflammatory cell profile in sputum, and exhaled breath condensate malondialdehyde (EBC-MDA). NFCB severity was assessed using BSI and FACED criteria. *Results*. Sputum neutrophil percentages inversely correlated with FEV1 ($P < 0.0001$; rho = −0.428), weakly with Leicester Cough Questionnaire score ($P = 0.068$; rho = −0.58), and directly with duration of the disease ($P = 0.004$; rho = 0.3) and BSI severity score ($P = 0.005$; rho = 0.37), but not with FACED. Sputum neutrophilia was higher in colonized subjects, *P. aeruginosa* colonized subjects showing greater sputum neutrophilia and lower FEV1. Patients with ≥3 exacerbations in the last year showed a significantly greater EBC-MDA than the remaining patients. *Conclusions*. Sputum neutrophilic inflammation and biomarkers of oxidative stress in EBC can be considered good biomarkers of disease severity in NCFB patients, as confirmed by pulmonary function, disease duration, bacterial colonization, BSI score, and exacerbation rate.

1. Introduction

Noncystic fibrosis bronchiectasis (NCFB) is a disease characterized by permanently dilated airways due to bronchial wall structural components destruction, as a result of bronchial inflammation caused by recurrent or chronic infections [1]. Clinical and functional features of NCFB are chronic cough, at times with haemoptysis, increased sputum production due to impaired mucus clearance, dyspnoea, and frequent bacterial colonization.

Neutrophils play a key role in the development and progression of bronchiectasis. Bronchial biopsies in patients with bronchiectasis have demonstrated tissue neutrophilia, a mononuclear cell infiltrate composed mainly of CD4+ T cells and CD68+ macrophages, and increased expression of IL-8 and other chemokines [2]. Neutrophil massive recruitment into the airways in response to infective or inflammatory triggers results in proteolytic enzymes such as neutrophil elastase (NE) and matrix metalloproteinases (MMP) release, leading to airway matrix destruction [3]. The damage of the epithelial layer is responsible for reduced mucociliary clearance efficiency, leading to bacterial colonization and perpetuating the vicious circle "bacterial load-inflammation-airway damage."

Systemic inflammation as demonstrated by increased blood neutrophils and plasma cytokines (like TNF-alfa) has been measured in patients with NCFB, with some relationship with disease severity and bacterial colonization [4]. Despite the relevant role the neutrophilic inflammation is believed to have in the progression of the disease, few

studies have evaluated the relationship between sputum inflammatory biomarkers and clinical or functional findings in patients with bronchiectasis [5–7]. In addition, very few data are reported on the measurement of oxidative stress biomarkers in exhaled air in this kind of patients [8].

The aim of this study was to evaluate the level of bronchial inflammation in subjects with stable NCFB and to compare it with clinical and functional findings in the attempt to understand its role in NCFB severity and progression. We used sputum inflammatory cell counts for confirming and extending previous data from the literature and EBC-MDA as a new tool in the correlation between biomarkers and clinical and functional findings.

2. Patients and Methods

We performed a cross-sectional study on 152 patients with noncystic fibrosis bronchiectasis (NCFB) presenting to our pulmonary unit. Criteria for entering in the study were the following: (a) NFCB diagnosis according to high resolution computed tomography (HRCT) [9], (b) absence of clinical, radiological, and laboratory tests suggestive of cystic fibrosis, and (c) stable phase of the disease (no acute exacerbations or antibiotic use in the previous 4 weeks). Diagnosis and assessment of NCFB have been done according to BTS Guidelines [10]. Patients with long-lasting clinical history of COPD (confirmed by spirometry) associated with smoking habit and bronchiectasis associated with interstitial lung diseases were not included.

In the same day, patients underwent complete spirometric evaluations (including measurement of diffusing capacity and bronchodilator reversibility), arterial blood gas analysis, Leicester Cough Questionnaire (LCQ, in 62 out of 152 patients) [11], assessment of the number and severity of comorbidities, assessment of the number of acute exacerbations in the last year (defined as increase in cough and/or sputum requiring a short course of antibiotics, in few cases also as fever and/or chest pain), sputum collection for bacterial colonization and inflammatory cell analysis, exhaled breath condensate (EBC) collection for malondialdehyde (MDA) assay, and exhaled Nitric Oxide (NO) measurement. Information about the number and the severity of exacerbations in the last year was collected. The aetiology of bronchiectasis was derived from the clinical history, CT features and from a set of blood analyses (including serum immunoglobulin, standard rheumatologic blood tests, and blood cell counts), while more detailed blood tests (like extensive rheumatologic evaluation, serum IgE or IgG for *Aspergillus*, and nasal biopsy) were limited to patients with some clinical suspect of specific diseases (like mucociliary dyskinesia, bronchopulmonary aspergillosis, and rheumatologic disorders). AAT1 was not systematically measured. Localization, extent, and type of bronchiectasis and presence of any inflammatory impairment/alteration due to other/different diseases were evaluated by HRCT performed in the last year before the study assessment [1, 12]. NCFB severity was assessed by both FACED score and Bronchiectasis Severity Index (BSI) [13, 14].

Pulmonary function testswere performed using Medical Graphics equipment (Elite Series Plethysmography, Medical Graphics, Saint Paul, USA), according to the ATS recommendations [15]. Arterial blood sample for the measurement of PaCO2 and PaO2 was taken in sitting position. Pharmacologic treatment with bronchodilators and/or inhaled corticosteroids (ICS) was stopped 2 days before spirometry, while long-term azithromycin treatment was withdrawn 4 weeks before the study day.

Informed signed consent was obtained from each patient. As an observational study, the study protocol had been notified to the local Ethic Committee of our University Hospital.

2.1. Sputum Induction and Analysis. Sputum collection and analysis were carried out according to a standardised protocol. In patients with spontaneous sputum production ($N = 35$), samples were collected for bacteriology and inflammatory cell count, after mouth rinsing with normal saline. In patients who did not expectorate spontaneously ($N = 74$), sputum production was induced with hypertonic saline solution (HS: NaCl 4.0% w/v) (2.8 mL/min output; Sirius, Technomed, Firenze, Italy), after inhaled salbutamol pretreatment. Nebulization was stopped after 15 min or earlier if FEV1 fell by 20% or more from baseline values [16].

Bacterial assessment of the spontaneous or induced sputum was performed according to the current standard laboratory methods. Gram-positive and Gram-negative bacteria, TB and non-TB mycobacteria, and fungi were searched.

For inflammatory cell counts, sputum samples were processed within two hours from collection; more dense portions were selected and processed as previously described [17]. At least 350 inflammatory cells were counted and macrophage, lymphocyte, neutrophil, and eosinophil values were expressed as percent of total inflammatory cells. Slides with cell viability <50% or an amount of squamous cells such that 350 inflammatory cells could not be counted were considered inadequate and discarded. Our reproducibility for sputum inflammatory cell count was previously assessed: excluding lymphocytes (RI: 0.15), it was considered as satisfactory: RI was 0.80 for macrophages, 0.85 for neutrophils, and 0.82 for eosinophils [18].

2.2. Exhaled Breath Condensate (EBC) Collection and MDA Analysis. EBC was collected by cooling exhaled air with a specially designed condenser (ECoScreen, Jaeger, Wurzburg, Germany). Subjects breathed tidally for 15 min through a two-way nonrebreathing valve by which inspiratory air and expiratory air are separated, and saliva is trapped [19]. The condensate obtained was immediately stored at −30°C until analysis. MDA in EBC was quantified, according to the method described by Lärstad et al. [20], using High-Performance Liquid Chromatography (HPLC) (Waters 1525) with fluorescence detector (Multi $\sqrt{}$ Fluorescence Detector, Waters 2475), after derivatization with thiobarbituric acid (TBA). The MDA-TBA adduct was detected using excitation and emission wavelength of 532 nm 553 nm, respectively. Our limit of detection was 6 nm/L, the intra- and interassay

reproducibility were 0.9 and 10.4%, respectively, and the recovery was 96% [21].

2.3. Exhaled Nitric Oxide (NO) Measurement. NO was measured in exhaled air, using a Nitric Oxide Analyzer (NOA 280, Sievers Instruments, Inc., USA). Patients performed a single slow exhalation (30–45 sec) through a mild resistance, while maintaining expiratory flow of about 50 L/min, and NO concentration at mouth level was registered all along the expiration phase. At least three acceptable maneuvers with NO variability lower than 10% were obtained, and the highest value was considered [22].

2.4. Statistical Analysis. Pulmonary function parameters are expressed as mean ± SD. Biological data are expressed as median and range. Differences between two groups were tested by means of *t*-test for normally and Mann-Whitney test for nonnormally distributed variables. For analysis of larger groups, ANOVA test, for normally distributed variables, and Kruskal-Wallis test, for nonnormally distributed variables, were used.

Correlations were evaluated by means of Spearman's rank correlation test. A P value lower than 0.05 was considered as significant.

3. Results

3.1. Characteristics of the Examined Patients. We studied 65 males and 87 females with NCFB, with a mean age of 62.6 years. Main demographic and clinical characteristics are reported in Table 1. Bronchiectasis was diffuse in 84% and localized in 10% of patients; 77% showed cylindric, 12% cystic, and 5.3% varicose bronchiectasis (in the remaining patients, a recent CT assessment was not available). NFCB aetiology was idiopathic in 56%, postinfectious in 27%, TB-related in 8%, and of other causes in 9% of patients (postsurgical BE: 2.7%, DCP: 1.4%, hypogammaglobulinemia: 2.0%, Churg-Strauss syndrome: 0.7%, Kartagener syndrome: 0.7%, medium lobe syndrome: 0.7%, and occupational exposure: 0.7%).

Patient showed mild airway obstruction, no hyperinflation, and normal diffusing capacity; 24 patients had a bronchodilator reversibility greater than 12%. Frequent exacerbations (mean value, 2.5 exacerbations/year) were reported by 56% of patients, while persistent cough (20,4%), dyspnoea on exercise (11%), and sputum production and/or hemoptysis (6%) were reported in a limited number of patients. LCQ questionnaire showed a mild impairment due to cough, as only 21 out of 62 patients reported a score >0. NCFB severity was mild according to FACED and moderate according to BSI. Presence of airway pathogens was observed in 70.5% of patients, and of these 52% were represented by *Pseudomonas aeruginosa*. Patients with *Pseudomonas aeruginosa* infection showed lower levels of FEV1 in comparison with patients with other or no colonization ($P = 0.007$).

Regular pharmacologic treatment was performed by 82 patients (54.3%) with inhaled corticosteroids at different doses (always associated with long-acting beta2-agonist and/or antimuscarinic drugs) and by 71 patients (47%) with long-term oral azithromycin (750–1000 mg weekly). Relevant comorbidities were reported by 49 out of 152 patients (32%), in particular cardiovascular diseases (29%), upper airway diseases (26%), and GI diseases (13%).

Table 1: Main clinical and functional data of the examined patients with NCFB.

Subjects, *n*	152
Male, *n* (%)	65 (42.7)
Age, years	62.6 ± 14
Smoking habit, yes/ex/no (%)	8/50/94 (5.3/32.9/61.8)
Pack/years	24.9 ± 18.8
BMI, Kg/m^2	24.9 ± 4.8
FEV1, % pred.	84.0 ± 21.2
FEV1/VC, % pred.	84 ± 14
TLC, % pred.	104 ± 15
DLCO SB, % pred.	86 ± 18
LCQ, mean	15.0 ± 4.0
BSI, mean	7.1 ± 3.3
FACED score, mean	2.0 ± 1.5
Airway pathogens, %	
Pseudomonas aeruginosa	36.6%
Staphylococcus aureus	10.7%
Aspergillus (gender)	3.6%
Non-TB mycobacteria	1.8%
Other	17.8%
No colonization	29.5%

Table 2: Biomarkers measured in the sputum, EBC, and exhaled air in the examined patients with NCFB.

Sputum inflammatory cells	
Neutrophils, median (range), %	79.3 (1.5–98.1)
Eosinophils, median (range), %	0.8 (0–70.2)
Exhaled NO, ppb	22.5 (2–168)
MDA (EBC), nM	30.4 (6–116)

Of 152 patients, 116 produced an adequate sputum sample. Inflammatory cell analysis showed a prevalent neutrophilic profile, with 69% of them having a sputum neutrophil percentage >64% [23]. Eosinophils percentages were equal to or more than 3% in only 20% of patients (Table 2). Patients with airway bacterial pathogens showed higher levels of sputum neutrophils than patients with no pathogens ($P = 0.009$), and those with *Pseudomonas aeruginosa* had even more sputum neutrophilia in comparison with those with other bacteria ($P = 0.006$). Mean levels of EBC-MDA and FeNO are also reported in Table 2. MDA levels were slightly higher than those previously reported in bronchiectasis (19.2 (6–54) nM) [21] and similar to those found in moderate COPD patients (29.1 (9–81) nM). Levels of FeNO >25 ppb were found in 25.5% of patients.

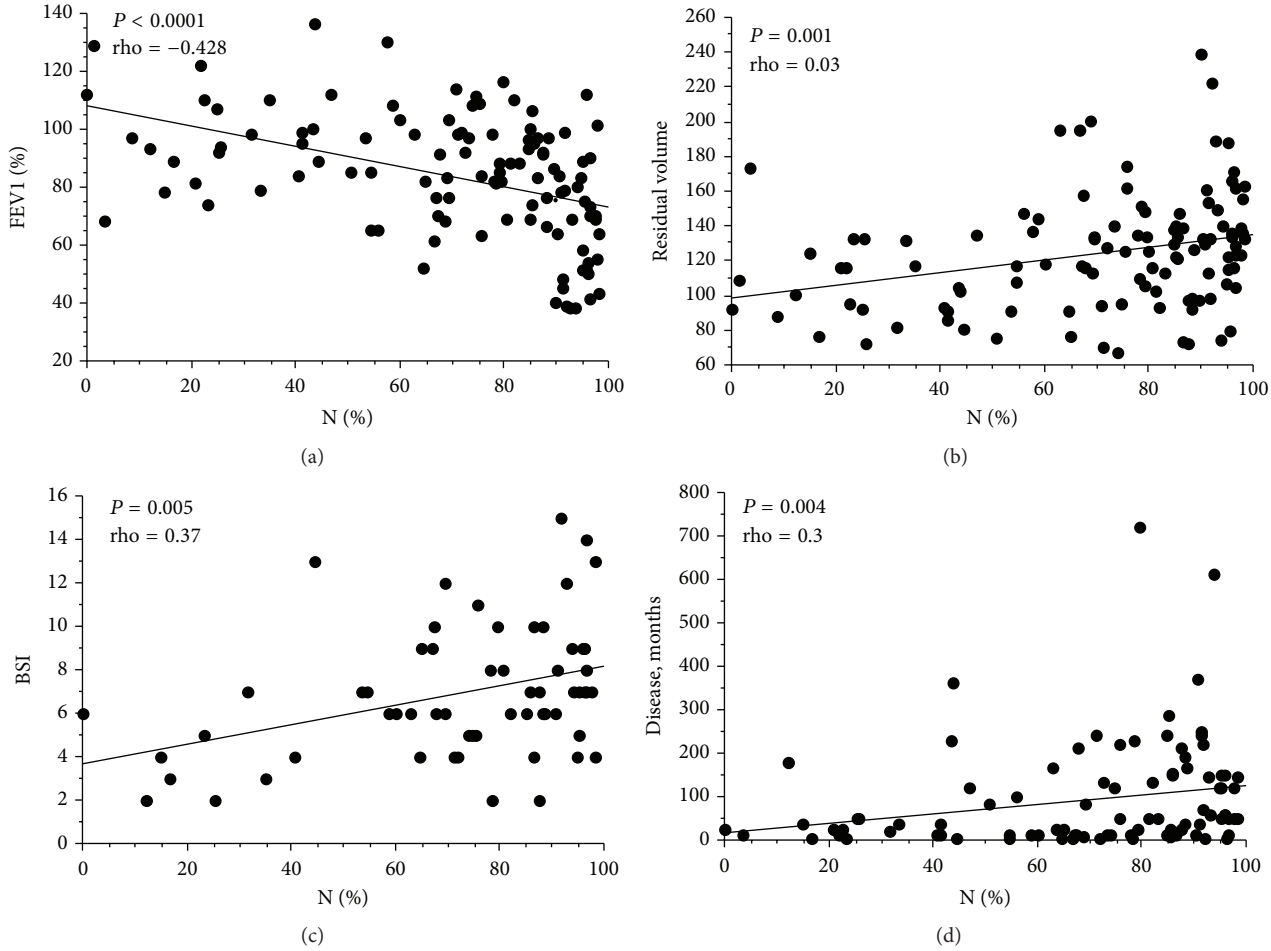

Figure 1: Correlation between neutrophils % in induced sputum and (a) FEV1, % predicted, (b) residual volume, (c) BSI, and (d) months between diagnosis of bronchiectasis and evaluation in the studied patients. Although these correlations are all statistically significant, the dispersion of the single data point is high.

3.2. Correlations. FEV1% predicted correlated with BMI ($P = 0.03$; rho = 0.178) and number of pulmonary lobes involved in the disease ($P = 0.039$; rho = −0.21).

Neutrophil percentages in induced sputum were inversely correlated with FEV1% predicted ($P < 0.0001$; rho = −0.428) and FEV1/VC ($P < 0.0001$; rho: −0.4), while sputum neutrophil percentages positively correlated with months between diagnosis and study evaluation ($P = 0.004$; rho = 0.3), residual volume ($P = 0.001$; rho = 0.03), and BSI severity score ($P = 0.005$; rho = 0.37), but not with FACED (Figure 1). Sputum neutrophil percentages were inversely correlated with FeNO ($P = 0.01$; rho = −0.28).

MDA levels in EBC significantly increased with the increasing number of exacerbations in the previous year ($P < 0.05$ by Kruskal-Wallis test) (Figure 2). In particular, patients with 3 or more exacerbations in the last year showed a significantly greater MDA concentration in EBC than patients with 1 or 2 exacerbations or patients without any exacerbations.

4. Discussion

In this study, the analysis of bronchial inflammatory biomarkers in induced sputum and exhaled air was aimed at a better characterization of adult patients with NCFB. We found that neutrophilia in induced sputum is a good marker of severity as confirmed by its correlation with several functional findings (FEV1, FEV1/VC, and RV), severity index (BSI), and symptoms (LCQ) assessment questionnaire and also with the time elapsed after diagnosis, suggesting that inflammation grows over time and might contribute to the progression of the disease. Moreover, neutrophilia was associated with the presence of airway pathogens, in particular with the presence of *Pseudomonas aeruginosa*.

The large majority of our patients (almost 70%) showed a percentage of sputum neutrophils greater than that observed in normal subjects [23], despite the fact that they were examined in a stable phase of the disease. This confirms that neutrophilic inflammation is a strong component of the disease. As reported by other authors [7, 24], sputum

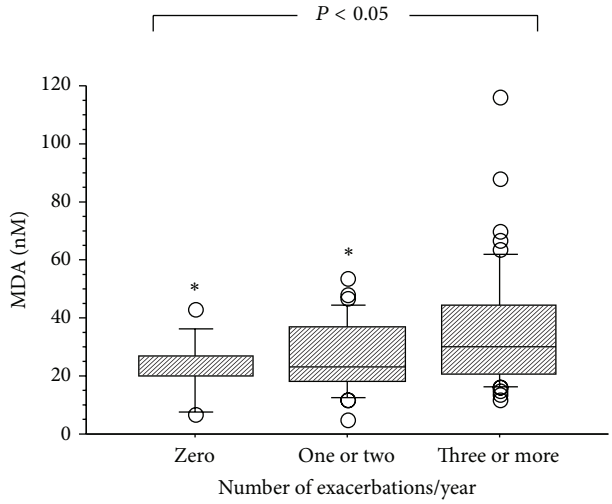

Figure 2: Box plots of malondialdehyde (MDA) in EBC according to the number of exacerbations in the preceding year. $^{*}P < 0.05$ by Dunn-Bonferroni post hoc test.

neutrophilia was associated with the type and severity of bacterial colonization, and also in our large group of patients, those with *Pseudomonas aeruginosa* had greater sputum neutrophilia and lower FEV1 in comparison with patients without airway pathogens or patients with other bacteria or fungi. In addition, we observed in 20% of our patients a sputum eosinophil percentage ≥3%, which is considered the upper limit of the normal distribution. To the best of our knowledge, this data has not been reported previously, and it can be due to the well-known association between bronchiectasis and asthma symptoms [25]. We did not find any relationship between the presence of sputum eosinophilia and acute FEV1 reversibility after salbutamol, clinical history of asthma, or treatment with inhaled corticosteroids (data not shown); other information regarding family history of allergic diseases, allergic sensitization, or bronchial hyperresponsiveness was not available in our patients. In any case, it is possible that this "asthmatic" component may play a role in the clinical manifestation of the disease and in the response to treatment.

Our study confirms and extends on a larger group of patients previous observations which have demonstrated the prominent neutrophilic systemic and airway inflammation in NCFB in comparison with control subjects and also some relationship between the level of inflammation and indices of severity of the disease, like pulmonary function, extension of bronchiectasis at HRCT, and bacterial colonization [4–8]. In addition to that, we found in our study new correlations not previously reported: in particular, sputum neutrophils directly correlated with residual volume, the duration of the disease, and its severity as assessed by the BSI score [14].

The inverse correlation between residual volume and sputum neutrophilia may suggest some involvement of small airways, leading to air trapping due to mucus plugging in the lower airways and then to a redistribution of lung volumes. This may be due to some potential extension of inflammation from airways of large or medium calibre to small airways, with potential consequences on the gas exchange.

We found a good correlation between sputum neutrophilia and duration of the disease, and also with BSI but not with FACED. In effect, BSI includes a larger number of variables than FACED and allows therefore a better stratification of the patients. In addition, FACED has not been validated and therefore it is uncertain whether it can be confidently used. To our knowledge, this is the first time that sputum neutrophilia has been related to this composite score assessing the severity of bronchiectasis, confirming that also this biomarker correlates with the comprehensive evaluation of disease severity as assessed by BSI.

Oxidative stress is a major component of the mechanisms leading to the damage of the airway wall in bronchiectasis [26] and also in other diseases, like COPD. In our study, MDA levels in EBC correlated with the number of exacerbations in the previous year. EBC-MDA concentrations in our patients were higher in comparison with a group of normal subjects studies in a previous methodologic paper [21]. MDA is a well-known biomarker of the oxidative stress, and we as well as other authors have demonstrated elevated MDA concentrations in EBC of patients with different inflammatory airway diseases [21, 27–29]. This means that MDA could have a role as indicator of future risk of exacerbation, potentially suggesting a different treatment strategy.

Our study has some limitations. Firstly, we did not include a control group of normal subjects; however, the difference between patients with NCFB and normal subjects as regards both systemic and airway inflammations is well known [4–8]. Secondly, we took into consideration only a single bacterial evaluation in the sputum for defining patients with bacterial colonization when repeated confirmations of the presence of sputum pathogens are required. In effect, in many of our patients, the presence of sputum pathogens has been confirmed in previous sputum specimens, and furthermore each patient was examined in a stable phase of the disease. Thirdly, some additional evaluations for explaining the presence of sputum eosinophilia in a subgroup of our patients were not included. Finally, we included both spontaneous and induced sputum in the inflammatory and microbiologic assessment; although these measurements are not interchangeable, previous studies have demonstrated similar inflammatory cell percentages in both samples [30]. On the contrary, this is one of the largest series of NCFB patients, examined in a very extensive way.

In conclusion, sputum neutrophilic inflammation in NCFB patients can be considered a good biomarker of disease severity, as confirmed by pulmonary function, disease duration, bacterial colonization, and BSI score.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Metalloproteinases and Their Tissue Inhibitors in Comparison between Different Chronic Pneumopathies in the Horse

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In chronic respiratory disease, matrix metalloproteinases (MMPs) contribute to pathological tissue destruction when expressed in excess, while tissue inhibitors of metalloproteinases (TIMPs) counteract MMPs with overexpression leading to fibrosis formation. They may be out of balance in equine pneumopathies and serve as biomarkers of pulmonary inflammation. We hypothesized that MMPs and TIMPs correlate to clinical findings and bronchoalveolar lavage fluid cytology in different equine chronic pneumopathies. Using a scoring system, 61 horses were classified controls as free of respiratory disease ($n = 15$), recurrent airway obstruction (RAO, $n = 17$), inflammatory airway disease (IAD, $n = 18$), or chronic interstitial pneumopathy (CIP, $n = 11$). Zymography and equine MMP and TIMP assays were used to detect MMP-2, MMP-8, MMP-9 as well as TIMP-1, and TIMP-2 in BALF supernatant. MMP-2, TIMP-1, and TIMP-2 concentrations were significantly increased in RAO and IAD compared to controls. MMP-9 concentration and MMP-8 activity evaluated by fluorimetry were significantly increased in RAO, IAD, and CIP. These results were confirmed by zymography for MMP-2 and MMP-9 activity in 52 horses. In conclusion, MMPs and TIMPs correlate well with clinical and cytologic findings. These findings support the usefulness of MMPs, TIMPs, and their ratios to evaluate the severity of respiratory disease and may help to identify subclinical cases.

1. Introduction

The extracellular matrix (ECM) represents the scaffold that supports the alveolar wall and has a major impact on lung architecture, homeostasis, and function. The pulmonary ECM underlays a continuous turnover; a dynamic equilibrium between synthesis and degradation of the ECM is maintained for physiological balance. This balance is controlled by synthesis and deposition of ECM components, proteolytic degradation of ECM by matrix metalloproteinases (MMPs), and inhibition of MMP activity by specific tissue inhibitors of matrix metalloproteinases (TIMPs) [1–3]. In health, MMPs degrade the ECM to allow normal tissue repair, but in chronic inflammation they contribute to pathological tissue destruction when expressed in excess [4]. Thus, it has been suggested that MMPs can either protect against or contribute to pathology in inflammatory processes by exacerbation of aberrant lung remodeling [5–7]. ECM degradation results in destruction of interstitial collagen and release of degraded collagen fragments, which results in neutrophil influx with the production of chemoattractants [8]. In chronic respiratory disease, remodeling results in decreasing airway lumen, increased smooth muscle mass, peribronchial fibrosis, epithelial cell hyperplasia, and impaired airway function [9–11]. Regulation of remodeling may be a key for developing new therapeutics and disease management [2].

Matrix metalloproteinases (MMPs) were first described over 50 years ago by Gross and Lapiere [12]. Collagenolytic MMP-8 was increased in tracheal epithelium lining fluid (TELF) of RAO affected horses [13]. Immunoreactivity of collagenases MMP-8 and MMP-13 was significantly increased in TELF of horses with RAO, compared to healthy horses, and was positively correlated with the amount of degradation of type-I collagen [14].

Markedly increased elastolytic activity in TELF was also found in RAO, suggesting participation of elastases (MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, and MMP-12) [15]. Other authors found no difference in pro-MMP-2 compared to healthy horses and suggested that MMP-2 may represent a housekeeping proteinase involved with normal tissue remodeling [16]. Previously it has been described that the molecular weight of pro-MMP-2 is 65–75 kDa and that of lower molecular weight gelatinolytic species is below 50 kDa [17]. In horses, MMP-9 is found elevated in RAO affected horses. In TELF and BALF MMP-9-related gelatinase-activity was represented by 5 bands: high molecular weight gelatinase complex (above 110 kDa), pro-MMP-9 (90–110 kDa), and active MMP-9 (75–85 kDa) [17]. In tracheal aspirates of RAO affected horses, mainly high molecular weight bands (150–210 kDa) and 90–110 kDa bands were found in symptomatic disease phases compared to healthy horses [16]. MMP-9 represents the largest and complex member of MMPs that is present in low quantities in the healthy adult lung but much more abundant in several lung diseases, including asthma, idiopathic pulmonary fibrosis, and RAO [18]. BALF gelatinolytic MMP activity in RAO affected horses increases as early as 5 hours after natural challenge and correlates with the BALF neutrophil counts [18, 19].

Tissue inhibitors of metalloproteinases are specific inhibitors of MMPs that bind to MMPs and inhibit their enzymatic activity. Four TIMPs have been identified including TIMP-1, TIMP-2, TIMP-3, and TIMP-4 and inhibit all MMPs tested [20, 21]. In human COPD, increased MMP-9 and TIMP-1 concentrations were detected in plasma and BALF [22].

TIMP-1 is the most widely distributed and acts on all active MMPs. A higher concentration of TIMP-1 was found in human BALF of asthmatic patients compared to healthy controls; thus it might be a better marker for mild asthma [23]. Also, high levels of TIMP-1 are associated with increased airway fibrosis. In addition, the molar concentration of TIMP-1 often exceeds the concentrations of MMP-9 and other MMPs [24]. These findings suggest that although TIMP-1 protects airway tissue from enhanced MMP activity, its increase may also be pathogenic and lead to enhanced airway fibrosis.

TIMP-2 appeared to be effective in preventing ECM damage by inhibition of MMP-2 and related proteolytic activity. Additionally, it serves as a target for therapy as reduced airway inflammation and hyperresponsiveness were observed after the administration of recombinant TIMP-2 in the bronchial tree [25].

In addition to MMP and TIMP concentrations, MMP : TIMP ratios have raised increasing interest in human asthma and COPD. They have been found to be even more sensitive biomarkers than MMPs and TIMPs. To our knowledge, TIMPs and MMP : TIMP ratios in equine pulmonary disease have not been studied so far.

While the role of MMPs in RAO has been studied intensively in the horse, not much is known about other chronic respiratory diseases leading to exercise insufficiency and therefore representing a major economic problem in the horse industry. We hypothesized that MMP-2, MMP-8 and MMP-9 as well as TIMP-1 and TIMP-2 concentrations correlate with clinical and cytologic data and increase in different forms of chronic pneumopathy including RAO, inflammatory airway disease (IAD), and chronic interstitial pneumopathy (CIP). We also suspected that MMP : TIMP might be valuable biomarkers in equine disease.

2. Materials and Methods

2.1. Preparticipation Examination. A total of 64 horses were examined, of which 15 had no clinical signs or history of respiratory disease and 49 were presented to the clinic with a history of chronic lower airway disease. Sampling of horses affected by respiratory disease was not classified as animal experiments by the State Office of Health and Social Affairs Berlin (LaGeSo); sampling of control horses was approved (reference number L0294/13). The owners gave permission to involve their horses in the study.

The preparticipation examination included anamnesis documentation, clinical examination, exercise test, blood gas analysis, bronchoscopy, BALF cytology, and thoracic radiography. A modified clinical score system including endoscopy results, parameters of gas exchange, and BALF cytology was used, shown in Table 1 [26, 27]. Additionally, results of thoracic radiography were included to classify horses as free of respiratory disease (controls), recurrent airway obstruction in exacerbation (RAO), inflammatory airway disease or RAO in remission (IAD), and chronic interstitial pneumopathy (CIP) or were excluded from the study, if they could not be assigned to these groups. In detail, groups were defined as follows:

(i) Controls: no history of respiratory disease, clinical score <2, no tracheal secretions, low cellular density and neutrophils ≤ 8% in BALF, $AaDO_2$ ≤8 mmHg, and exclusion of acute signs of infection (leukocytosis, fever, and depression).

(ii) RAO: history of recurrent cough or dyspnea, clinical score >6, high amount or viscosity of tracheal secretions, high cellular density and neutrophils ≥25% in BALF, $AaDO_2$ >8 mmHg, and exclusion of acute signs of infection (leukocytosis, fever, and depression) according to Robinson [28].

(iii) IAD: history of cough or exercise insufficiency, clinical scores 2–6, low to moderate amount or viscosity of tracheal secretions, increased cellular density and neutrophils ≥8% or mast cells ≥2% or eosinophils ≥0.1% in BALF, and exclusion of acute signs of infection (leukocytosis, fever, and depression) according to Couëtil et al. [29].

(iv) CIP: history of exercise insufficiency, clinical score 2–6, low to moderate amount or viscosity of tracheal secretions, increased cellular density and ratio of macrophages: neutrophils ≥2.5 : 1 in BALF, increased interstitial opacity of thoracic radiographs, and exclusion of acute signs of infection (leukocytosis, fever, and depression) according to Dieckmann et al. [30].

Table 1: Clinical scoring system, modified from Ohnesorge et al. (1998) [26] and Gehlen et al. (2008) [27].

		Score	Max. points
(1) Cough induction	No cough after manual compression of larynx	0	1
	Coughing during manual larynx compression	1	
	Very frequent coughing	1	
	Spontaneous coughing	1	
(2) Dyspnoea at rest	Prolonged expiration	1	3
	Abdominal breathing	1	
	Sinking of the intercostal area	3	
	Nostril flare	3	
	Heaves line	3	
	Anal pumping	3	
(3) Lung percussion	3 fingers	0	2
	Handbreadth	1	
	Damping	2	
(4) Lung auscultation	Rattling	2	2
	Crackle	2	
	Wheezing	2	
(5) Endoscopy	Significantly increased secretions with moderate viscosity	1	2
	Highly increased secretions with high viscosity	2	
	Thickened carina of the trachea	1	
(6) BALF cytology	Neutrophils <8%	0	3
	Neutrophils 8–15%	1	
	Neutrophils 15–25%	2	
	Neutrophils >25%	3	
(7) Arterial blood gas analysis	$AaDO_2$: 0–7 mmHg	0	2
	$AaDO_2$: 7–14 mmHg	1	
	$AaDO_2$: >14 mmHg	2	

2.2. BALF Collection and Processing. During endoscopy, 20 mL of 2% lidocaine (Lidocaine, Bela-Pharm GmbH, Vechta, Germany) was infused around the tracheal bifurcation. The catheter (Silicone Bronchoalveolar Lavage Catheter 300 cm, Smiths Medical ASD, Inc., USA) was wedged into the bronchus by mean of an air balloon. Five hundred milliliters of prewarmed phosphate buffered saline (phosphate buffered saline, Lonza, Verviers, Belgium) was infused as recommended by the International Workshop on Equine Chronic Airway Disease [28] and immediately aspirated.

BALF was divided into 2 portions for cytological and biochemical examination. After centrifugation (Table Top Refrigerated Centrifuge Hermle Z326K, Hermle Labortechnik GmbH, Germany) at 1500 rpm for 10 min at 4°C the cell-free supernatant was stored at −80°C until being assayed. Cytology was performed using Wright-Giemsa staining and counting 500 cells at 500x magnification.

2.3. Gelatin Zymography (MMP-2 and MMP-9). Zymography was performed (gelatin zymogram gels (Life Technologies, USA); electrophoresis device XCell, Novex Experimental Technology, Japan) according to the manufacturer's manual. Human MMP-2 and MMP-9 controls (recombinant human MMP-2, USCN Life Science, Inc., China) were used together with a multicolor broad protein range protein ladder (Spectra Multicolor Broad Range Protein Ladder, Thermo Scientific, Rockford, USA) as control on each gel. Also, a sample of a control horse was applied to each gel to compare the signals to affected horses. Gels were scanned for digital analysis by densitometry using digital image analyzing software (ImageJ v1.47, Wayne Rasband, NIH, USA) for objective quantification of bands and the results were presented based on peak area [31].

2.4. ELISA of MMP-2, MMP-9, TIMP-1, and TIMP-2. The ELISAs used in this study were equine specific sandwich enzyme immunoassays for quantification of MMP and TIMP concentrations (equine MMP-2 kit, USCN Life Science, Inc., China; equine MMP-9 kit, USCN Life Science, Inc., China; equine TIMP-1 kit, USCN Life Science, Inc., China). Standards and samples were set up in duplicate according to the manufacturer's protocol. The absorbance was measured with an ELISA microplate reader (equine TIMP-2 kit, USCN Life Science, Inc., China) at 450 nm immediately. Calculation of the unknown sample concentration was made by inverted standard curve using the *Excel* software program.

2.5. Fluorimetry MMP-8 Assay. The MMP-8 assay (ELISA microplate reader, BioRad Laboratories, USA) was performed according to the manufacturer's protocol. Negative

Table 2: Results of clinical examinations. $*$ shows significant increase to controls at $p < 0.05$ and $**$ significant increase in RAO compared to IAD and CIP. RAO: recurrent airway obstruction, IAD: inflammatory airway disease, and CIP: chronic interstitial pneumopathy.

	Controls ($n = 15$)	RAO ($n = 17$)	IAD ($n = 18$)	CIP ($n = 11$)
Endoscopy score	0 ± 0	$1.82 \pm 0.39^{*}$	$1.17 \pm 0.71^{*}$	$0.91 \pm 0.7^{*}$
BAL score	0 ± 0	$3 \pm 0^{*}$	$1.39 \pm 1.04^{*}$	0.45 ± 0.69
Blood gas score	0.2 ± 0.4	$0.76 \pm 0.75^{*}$	0.17 ± 0.38	0.36 ± 0.81
Total exam score	0.27 ± 0.46	$8.12 \pm 2.23^{*,**}$	$3.88 \pm 1.41^{*}$	$2.36 \pm 2.66^{*}$

Table 3: Results of endoscopic examination. $*$ shows significant increase to controls at $p < 0.05$. RAO: recurrent airway obstruction, IAD: inflammatory airway disease, and CIP: chronic interstitial pneumopathy.

	Amount of secretions	Viscosity of secretions	Tracheal bifurcation
Controls ($n = 15$)	0.47 ± 0.64	0.4 ± 0.51	0.25 ± 0.45
RAO ($n = 17$)	$3.5 \pm 0.63^{*}$	$3.88 \pm 0.5^{*}$	$1.36 \pm 1^{*}$
IAD ($n = 18$)	$2 \pm 1.33^{*}$	$2.33 \pm 1.4^{*}$	$1.33 \pm 0.82^{*}$
CIP ($n = 11$)	$2 \pm 1.2^{*}$	$1.8 \pm 1.2^{*}$	$1.56 \pm 0.88^{*}$

controls containing assay buffer and positive controls using recombinant human purified MMP-8 were included.

2.6. Statistical Analysis. Data were statistically analyzed using SPSS (Sensolyte 520 MMP-8 Assay Kit, Anaspec, Inc., Fermont, USA) and expressed as mean ± standard deviation (SD). The data were tested for normal distribution using the Shapiro Wilks Test. While some data were found to be normally distributed, other was not, so we preferred nonparametric tests for the whole data. The level of significance was set at $p < 0.05$.

Kruskal Wallis H test was used to compare between controls and different disease groups followed by post hoc testing using Mann-Whitney U Test for 2-group comparison to determine intergroup differences.

Spearman rank correlation coefficients were calculated between clinical examination scores and blood gas scores and between these variables and the total examination score, neutrophil percentages, MMP-2, MMP-9 concentrations, and MMP-8 activity. The Spearman correlation coefficients were interpreted using the scale provided by Salkind, where the values between 0.8 and 1.0, 0.6 and 0.8, 0.4 and 0.6, 0.2 and 0.4, and 0.0 and 0.2 were defined as very strong, strong, moderate, weak, and very weak or no relationship, respectively [32]. The same was performed for calculated MMP : TIMP ratios.

3. Results

According to the results of the clinical examination, the 64 horses (35 geldings, 29 mares, aged 12.74 ± 5.25 years, BDW 473.79 ± 5.26 kg) presented for participation in this study were classified as follows: 15 horses (23.4%) were classified as free of respiratory disease (controls), 17 (26.6%) as RAO in exacerbation, 18 (28.1%) as RAO in remission or IAD, and 11 (17.2%) as CIP and 3 horses (4.7%) suffered from acute respiratory infections and were excluded. The overall results of the clinical examinations are presented in Table 2.

3.1. Endoscopy. The results of the endoscopic examination including the amount and viscosity of secretions and the tracheal bifurcation appearance were scored according to Dieckmann and Deegen [33] and Gerber et al. [34] and included into a modified overall clinical score [26, 27]. The results are presented in Table 3.

3.2. Pulmonary Function. The results of arterial blood gas analysis are presented in Table 4. PaO_2 and $AaDO_2$ were significantly increased in RAO but not in other disease groups.

3.3. BALF Cytology. Percentages of macrophages, lymphocytes, mast cells, and in particular neutrophils were highly and significantly different between controls and different disease groups (Table 5). BALF neutrophils percentage (reference range 0–8%) was significantly increased in RAO (60.68 ± 21.59%), IAD (15.64 ± 8.19%), and CIP (8.73 ± 5.71%) compared to controls (3.02 ± 2.41%). Also, BALF neutrophils percentage was significantly increased in RAO compared to IAD and CIP.

3.4. MMP-2 ELISA. In RAO (5.21 ± 0.77 ng/mL) and IAD (7.67 ± 15.5 ng/mL) highly significant increases in MMP-2 concentrations were found compared to controls (2.49 ± 0.83 ng/mL). MMP-2 was not increased in CIP (2.81 ± 0.34 ng/mL). Horses diagnosed with IAD showed a highly significant increase compared to RAO and CIP.

3.5. MMP-9 ELISA. Obvious differences between disease groups were detected. Highly significant increases in MMP-9 concentration were found in RAO (433.34 ± 89.05 pg/mL), IAD (312.06 ± 23.92 pg/mL), and CIP (263.2 ± 23.85 pg/mL) compared to controls (176.29 ± 60.22 pg/mL). In RAO a highly significant increase compared to IAD and CIP was evident. Other intergroup differences were not found significant.

Table 4: For arterial blood gas analysis, the results are expressed as mean ± SD. ∗ shows significant difference to controls at $p < 0.05$. RAO: recurrent airway obstruction, IAD: inflammatory airway disease, and CIP: chronic interstitial pneumopathy.

	$PaCO_2$ [mmHg]	PaO_2 [mmHg]	$AaDO_2$ [mmHg]
Controls ($n = 15$)	43.87 ± 2.53	101.95 ± 6.18	0.52 ± 1.03
RAO ($n = 17$)	43.63 ± 4.76	87.9 ± 12.15*	10.91 ± 9.3*
IAD ($n = 18$)	43.75 ± 3.02	94.98 ± 7.38	5.17 ± 8.03
CIP ($n = 11$)	44.19 ± 3.24	94.23 ± 9.4	5.55 ± 8.5

Table 5: For BAL cytology, the results of cell percentages are expressed as mean ± SD. ∗ shows significant differences to controls at $p < 0.05$, ∗∗ significant increase in RAO compared to IAD and CIP, and ∗ ∗ ∗ significant decrease in RAO compared to IAD and CIP. RAO: recurrent airway obstruction, IAD: inflammatory airway disease, and CIP: chronic interstitial pneumopathy.

	Macrophages [%]	Lymphocytes [%]	Neutrophils [%]	Eosinophils [%]	Mast cells [%]
Controls ($n = 15$)	56.48 ± 4.75	38.15 ± 6.41	3.02 ± 2.41	0.13 ± 0.27	2.22 ± 2.06
RAO ($n = 17$)	19.64 ± 12.07*,***	18.66 ± 12.16*	60.68 ± 21.59*,**	0.27 ± 0.35	1.16 ± 1.22
IAD ($n = 18$)	43.78 ± 12.98*	34.63 ± 13.65	15.64 ± 8.19*	1.95 ± 3.9	3.8 ± 3.21
CIP ($n = 11$)	50.83 ± 15.46	34.47 ± 11.87	8.73 ± 5.71*	0.95 ± 0.92	5.03 ± 4.34

3.6. MMP-8 Activity. MMP-8 activity showed significant differences between all groups. In RAO (21,802.03 ± 21,047 RFU), IAD (5,366.17 ± 1,434 RFU), and CIP (3,800.36 ± 403 RFU) significant increases were found compared to controls (3,556.63 ± 176 RFU). Intergroup analysis revealed highly significant increases in MMP-8 activity in RAO compared to IAD and CIP. A significant increase was also found in IAD compared to CIP.

3.7. TIMP-1 ELISA. Highly significant increases in TIMP-1 concentrations were found in RAO (328.19 ± 62.83 pg/mL) and IAD (308.92 ± 8.24 pg/mL) compared to controls (117.54 ± 45.62 pg/mL). Intergroup differences were not significant.

3.8. TIMP-2 ELISA. TIMP-2 concentrations were also highly significantly increased in groups RAO (27.75 ± 5.08 ng/mL) and IAD (25.42 ± 1.38 ng/mL) compared to controls (18.06 ± 2.37 ng/mL). Intergroup differences were not significant.

3.9. MMP-TIMP Ratios. Significant differences were found for RAO in MMP-9/TIMP-2, MMP-8/TIMP-1, and MMP-8/TIMP-2 ratios, for IAD in MMP-2/TIMP-2 and MMP-8/TIMP-1 ratios, and for CIP in the MMP-8/TIMP-1 ratio.

A concluding summary of all MMP- and TIMP-measurements is presented in Table 6 and for MMP/TIMP ratios in Table 7.

3.10. Gelatin Zymography. Gelatin zymography was performed on 52 BALF samples from controls ($n = 13$), RAO ($n = 17$), IAD ($n = 14$), and CIP ($n = 8$) affected horses. Gelatinolytic activity bands were detected at about 70 kDa for MMP-2 (pro-MMP-2) and at 100 and 140 kDa for MMP-9, respectively (pro-MMP-9 and high molecular weight forms). An example of a zymographic gel is shown in Figure 1.

Based on peak areas, high molecular weight bands of MMP-9 showed significant differences between groups. In RAO (10,967.31 ± 9,530.07) highly significant increases in peak areas were found compared to controls (619.29 ± 996.32) and also showed a highly significant increase compared to IAD (1,832.16 ± 2,111.29) and CIP (864.06 ± 767.93). Other intergroup differences were not found significant.

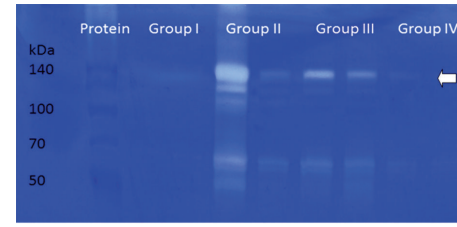

Figure 1: Gelatin zymography of MMP-2 and MMP-9. Examples of healthy controls (group I), RAO (group II), IAD (group III), and CIP (group IV). The 70 kDa bands are representative of pro-MMP-2 and those at 140 kDa are representative of high molecular weight MMP-9 (arrow) as checked in a comparison of protein marker and human MMP-2 and MMP-9 (data not shown).

Digital analysis for MMP-2 was based on bands of the gelatinolytic pro-MMP-2. Bands showed highly significant differences between RAO (17,288.53 ± 8,927.59) and IAD (3,530.94 ± 2,894.15) compared to controls (1,114.76 ± 672.72). Peak areas were significantly increased in RAO compared to IAD and CIP (2,799.45 ± 2,592.28, Figure 1). Other intergroup differences were not significant.

3.11. Correlation of MMPs, TIMPs and Clinical Score. The total clinical examination score showed a positive correlation with the concentrations of MMP-2 ($r = 0.75$), MMP-8 ($r = 0.77$), MMP-9 ($r = 0.79$), TIMP-1 (0.65), TIMP-2 (0.72), MMP-8 : TIMP-1 (0.76), and MMP-8 : TIMP-2 ratio (0.90). Also, a positive correlation was found with the MMP-2 ($r = 0.80$) and MMP-9 ($r = 0.71$) activity measured by gelatin zymography. All of these correlations had a level of significance of $p < 0.01$.

Table 6: MMP-2, MMP-9, TIMP-1, TIMP-2 ELISA, and MMP-8 fluorimetry measurements. The results are expressed as mean ± SD. ∗ shows significant increases to controls at $p < 0.05$, ∗∗ significant increase in IAD compared to RAO and CIP, and ∗ ∗ ∗ significant increase in RAO compared to IAD and CIP. RAO: recurrent airway obstruction, IAD: inflammatory airway disease, and CIP: chronic interstitial pneumopathy.

	MMP-2 [ng/mL]	MMP-9 [pg/mL]	MMP-8 [RFU]	TIMP-1 [pg/mL]	TIMP-2 [ng/mL]
Controls ($n = 15$)	2.49 ± 0.83	176.29 ± 60.22	3,556.63 ± 176	117.54 ± 45.62	18.06 ± 2.37
RAO ($n = 17$)	5.21 ± 0.77*	433.34 ± 89.05*,***	21,802.03 ± 21,047*,***	328.19 ± 62.83*	27.75 ± 5.08*
IAD ($n = 18$)	7.67 ± 15.5*,**	312.06 ± 23.92*	5,366.17 ± 1,434*	308.92 ± 8.24*	25.42 ± 1.38*
CIP ($n = 11$)	2.81 ± 0.34	263.2 ± 23.85*	3,800.36 ± 403*	205.47 ± 97.63	21.19 ± 2.45

Table 7: MMP : TIMP ratios. ∗ shows significant differences to controls at $p < 0.05$. RAO: recurrent airway obstruction, IAD: inflammatory airway disease, and CIP: chronic interstitial pneumopathy.

	MMP-2 : TIMP-1	MMP-2 : TIMP-2	MMP-9 : TIMP-1	MMP-9 : TIMP-2	MMP-8 : TIMP-1	MMP-8 : TIMP-2
Controls ($n = 15$)	0.021	0.137	1.500	9.761	30.259	196.934
RAO ($n = 17$)	0.016	0.188	1.320	15.62*	66.431*	785.668*
IAD ($n = 18$)	0.025	0.302*	1.010	12.28	17.37*	211.100
CIP ($n = 11$)	0.014	0.103	1.281	12.421	18.496*	179.35

3.12. Correlations of MMPs, TIMPs and BALF Cytology. BAL neutrophil percentages showed a positive correlation with the concentrations of MMP-2 (0.77), MMP-8 (0.76), MMP-9 (0.81), TIMP-1 (0.65), TIMP-2 (0.71), MMP-8 : TIMP-1 (0.90), and MMP-8 : TIMP-2 ratio (0.98). Also, a positive correlation was found with the MMP-2 (0.78) and MMP-9 (0.67) activity measured by gelatin zymography. All of these correlations had a level of significance of $p < 0.01$.

4. Discussion

In horses, collagenolytic and elastolytic MMPs in pulmonary secretions have been shown to increase during RAO [13–16]. In the present study, increased concentrations were found not only in RAO but also in IAD and chronic interstitial pneumopathies by semiquantitative and quantitative measurements and were highly correlated with the results of the clinical examinations and BALF cytology. TIMP concentrations were also increased in RAO and IAD but not in CIP. Healthy horses seem to have minimal gelatinolytic and collagenolytic activities, as MMP activity is physiologically balanced by TIMPs. An imbalance between MMP expression, activation, and inhibition is associated with tissue destruction in inflammatory lung diseases.

In the present study, MMP-2 and MMP-9 were identified using gelatin zymography as described previously in equine RAO [13, 17]. Human MMP markers served as controls due to unavailability of the purified equine protein. Although densitometry was used for quantification of the bands and the results were calculated based on peak area [31], we also aimed for direct quantitative measurements using equine ELISA kits. Quantification revealed the highest MMP-8 and MMP-9 activities in RAO, but activities in IAD and CIP were also significantly increased compared to controls.

In a study on tracheal epithelium lining fluid, the concentration of autoactive collagenase was approximately 7 times greater in RAO [14]. The authors concluded that collagenases are also involved in airway remodeling during exacerbation. Several studies have shown that MMP-9 is the main MMP present in the airways of RAO-susceptible horses following inhalation of hay dust or its components [19, 35, 36].

In unison with previous studies [15–17], our results of this study support the role of MMP-9 in RAO but also suggest a role of collagenases in equine IAD and CIP. The high positive correlations of MMPs and neutrophil percentages in BALF suggest these cells to be the origin of MMPs, in particular MMP-9, in RAO. Much debate exists on a possible precursor role of IAD for the development of RAO [29]. Increased MMP-8 activity in both groups may further support this theory. In addition, it seems possible that CIP may develop from IAD, which has a high prevalence in young sports horses, while RAO and CIP are more common in older individuals. In our study, we also found a correlation of MMP-8 concentrations and neutrophil percentage in BALF samples. Increased collagenase activity in lungs of humans with emphysema and bronchiectasis is suspected as a result of MMP-8 activity [37, 38]. All immunoreactive forms of MMP-8 detected in TELF samples were also detected in equine neutrophil lysate. Therefore, neutrophil-derived MMP-8 species were suggested to be the cause of the MMP-8 immunoreactivity detected in TELF samples [14]. Immunoreactivity for MMP-8 in TELF from RAO horses was approximately 13 times greater than in controls [14]. The factor between RAO horses and controls is even larger in our study and there were also significant increases in IAD and CIP. Nevertheless, the highest concentrations were measured in RAO with significant differences to the other groups.

The role of MMP-2 is of controversy in the literature. MMP-2 has been considered to be constitutively expressed [14, 35] and therefore its induction in inflammation has rarely been detected. Our results support this theory, as zymography only revealed bands of pro-MMP-2 in most cases. The MMP-2 ELISA also showed the highest values in horses suffering from IAD, which is characterized by a lower grade of inflammation and pulmonary dysfunction compared to RAO in exacerbation. In addition, no MMP-2

increase was found in CIP; therefore a constitutive role for this enzyme remains likely.

In men, increases in TIMP-1 and TIMP-2 have been found in asthma and COPD. Results of the presented study show that the same is true for equine RAO and IAD, but not for CIP. This makes sense, as CIP represents the final stage of interstitial pneumonia, characterized by a low grade of inflammation and organized fibrotic tissue. High correlations of MMPs as well as TIMPs with clinical findings and BALF cytology over all horses substantiate this result. Despite these obviously clear results, MMP : TIMP ratios raised our special interest, as they show a possible disbalance between ECM degradation and repair mechanisms leading to either pulmonary tissue destruction or fibrosis formation in the end. Perhaps the most interesting one was the MMP-8 : TIMP-1 ratio, in which significant differences were found for all pneumopathies studied compared to healthy controls, but in different orientations. While RAO was characterized by predominating collagenolytic activity demonstrated by an increased ratio, IAD/RAO in remission and CIP were characterized by predominating fibrosis formation demonstrated by a decreased ratio. This shows that even forms of equine respiratory disease going along with very slight clinical signs and cytologic findings lead to fibrosis formation affecting prognosis in these patients. Additionally, the MMP-8 : TIMP-1 ratio can identify cases of respiratory disease, in which clinical signs and cytologic findings are almost unremarkable, which is very helpful, when examining horses in remission.

Equine RAO shows features of human asthma and COPD. While the recurrent character of the disease resembles asthma, long-term changes include irreversible remodeling of the bronchial wall leading to decreased gas exchange and pulmonary function as known for COPD. In asthma, the degree of MMP activity can be linked to intensity of the inflammatory processes in the airways; therefore the MMPs/TIMPs balance is widely accepted to have a role in the pathogenesis of airflow limitation and reflect the extent of structural changes in the lung [39–41]. Mediators released by activated parenchymal and inflammatory cells could induce MMPs secretion and activation, increase in MMP-9 activity, and elevated MMP-9/TIMP-1 ratios as demonstrated in mild asthma after allergen challenge in sputum and BALF [23, 42]. Specific allergen challenge is also able to induce changes in MMPs and TIMPs, in particular MMP-9, in occupational asthma [43]. In severe asthma, increased basal levels of MMP-9 were even observed in plasma [44]. No difference was also found for MMP-2, again supporting a constitutive role as discussed earlier.

Equine RAO also shows features of human COPD, in which long-term exposure to cigarette smoke, toxic gases, and particulate matter leads to airflow limitation and pulmonary failure [39]. As RAO the disease is characterized by an excess of extracellular matrix deposition in bronchial walls, known as remodeling and involving many members of the MMP family, chronic cough, and dyscrasia. Increased MMP-1 and MMP-9 levels have been detected in BALF of emphysema patients [38]. COPD patients show increased activities of MMP-2 and MMP-9 in their lung parenchyma [45] and increased gelatinolytic activity linked to MMP-2 and MMP-9 in their sputum [46, 47]. An increase of collagenolytic activity, probably due to elevated levels of MMP-8, was also found [48].

Chronic interstitial pneumopathy is a poorly defined disease in equine medicine. Descriptions in the literature are rare [49]. Dieckmann et al. [30] gave some definition criteria from the examination of 12 affected patients and Venner et al. [50] studied horses after experimental induction of acute interstitial pneumopathy. In the early stage of human lung fibrosis, gelatinolytic activity of MMP-9 seems predominant and probably contributes to disruption of alveolar epithelial basement membrane and enhances fibroblast invasion to alveolar spaces [39], while, in the late stages of the disease, MMP-2 seems to become predominant. The expression of the two gelatinases at different stages of fibrosis suggests that MMP-9 could be rather linked to inflammation-induced tissue remodeling, while MMP-2 may be associated with an impaired tissue remodeling leading to pathological collagen deposition and interstitial fibrosis [41].

A weak point of this study was group definition, as samples were obtained from clinic patients. Although IAD and RAO in remission were planned as two distinct groups, it was not possible to differentiate clearly between these two. Anamnestic information of respiratory distress was often unreliable and the majority of owners did not agree to a natural challenge test. Descriptions of equine CIP are rare in the literature and an international consensus statement is missing, so definition of this group was based on a quite old clinical case series including only 12 horses [30]. Thoracic radiography showing interstitial patterns is not very specific for CIP and may also be found in RAO [51] and IAD [52]. Again, the majority of owners did not agree to lung biopsies. We tried to face these problems by calculating correlations between MMPs and TIMPs with clinical and cytologic parameters over all 61 horses and found significant results for almost all correlations, demonstrating the value of MMPs, TIMPs, and MMP : TIMP ratios as biomarkers independent of diagnosis.

In conclusion, metalloproteinases and their inhibitors, in particular MMP-9 and TIMP-1, are increased in different chronic pneumopathies in the horse and correlate significantly with clinical and cytologic findings. MMPs, TIMPs, and in particular the MMP-8 : TIMP ratios are useful to evaluate the severity and character of respiratory disease and may have prognostic value for equine pneumopathies. Further studies should focus on the balance between MMPs and TIMPs and their progression during disease and possible improvement during therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ann Kristin Barton and Tarek Shety contributed equally to paper.

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Research Article

Correlation Network Analysis Reveals Relationships between MicroRNAs, Transcription Factor *T-bet*, and Deregulated Cytokine/Chemokine-Receptor Network in Pulmonary Sarcoidosis

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Sarcoidosis is an inflammatory granulomatous disease with unknown etiology driven by cytokines and chemokines. There is limited information regarding the regulation of cytokine/chemokine-receptor network in bronchoalveolar lavage (BAL) cells in pulmonary sarcoidosis, suggesting contribution of miRNAs and transcription factors. We therefore investigated gene expression of 25 inflammation-related miRNAs, 27 cytokines/chemokines/receptors, and a Th1-transcription factor *T-bet* in unseparated BAL cells obtained from 48 sarcoidosis patients and 14 control subjects using quantitative RT-PCR. We then examined both miRNA-mRNA expressions to enrich relevant relationships. This first study on miRNAs in sarcoid BAL cells detected deregulation of *miR-146a*, *miR-150*, *miR-202*, *miR-204*, and *miR-222* expression comparing to controls. Subanalysis revealed higher number of *miR-155*, *let-7c* transcripts in progressing ($n = 20$) comparing to regressing ($n = 28$) disease as assessed by 2-year follow-up. Correlation network analysis revealed relationships between microRNAs, transcription factor *T-bet*, and deregulated cytokine/chemokine-receptor network in sarcoid BAL cells. Furthermore, *T-bet* showed more pronounced regulatory capability to sarcoidosis-associated cytokines/chemokines/receptors than miRNAs, which may function rather as "fine-tuners" of cytokine/chemokine expression. Our correlation network study implies contribution of both microRNAs and Th1-transcription factor *T-bet* to the regulation of cytokine/chemokine-receptor network in BAL cells in sarcoidosis. Functional studies are needed to confirm biological relevance of the obtained relationships.

1. Introduction

Pulmonary sarcoidosis is an inflammatory disorder of unknown etiology characterized by the accumulation of activated Th1/Th17 lymphocytes and macrophages in the alveoli and subsequent granuloma formation [1–3]. The key role in the pathogenesis of sarcoidosis is played by proinflammatory cytokines and chemokines, molecules crucially involved in the activation of immune and inflammatory cells and their trafficking to the site of disease [4]. However, there is still limited information about the regulation of the cytokine/chemokine-receptor network in pulmonary sarcoidosis and its phenotypes.

There is a growing body of evidence that the regulation of inflammatory response is a very complex process involving coordinated participation of multiple regulation systems, such as an integrated network of microRNAs (miRNAs) and transcription factors [5, 6]. The emerging role of

miRNAs, a class of single-stranded noncoding RNAs of 19–25 nucleotides in length, in regulation of inflammatory response has been already reported in chronic pulmonary diseases such as asthma [7] and chronic obstructive pulmonary disease [8]. In sarcoidosis, altered miRNA pattern has been reported in lung tissues [9], peripheral blood mononuclear cells [9–11], and serum [10]. However, there is no information regarding the miRNA pattern in bronchoalveolar lavage (BAL) cells and their regulatory capability related to cytokine/chemokine-receptor network in pulmonary sarcoidosis. Also, a Th1-transcription factor *T-bet* has emerged as key regulator of crucial immune genes such as interferon-(IFN-) γ and chemokine receptor CXCR3 in sarcoid inflammation [12–14] as well as in other inflammatory conditions [15–17]. However, no information about the possible cooperation of this Th1-transcription factor and inflammation-related microRNAs in regulation of cytokine/chemokine-receptor network in BAL cells in sarcoidosis exists yet.

In this study, we aimed to investigate the gene expression pattern of candidate inflammation-related miRNAs in BAL cells obtained from sarcoidosis patients and control subjects. In order to assess the possible contributions of miRNAs as posttranscriptional regulators and *T-bet* as a driver Th1-transcription factor on sarcoid inflammation, we searched for relationships between miRNAs and *T-bet* with sarcoidosis-associated cytokine/chemokine-receptor network in BAL cells obtained from patients with sarcoidosis and subgroups with progressing and regressing disease as assessed by 2-year follow-up. We believe that understanding the transcriptional and posttranscriptional regulation of cytokine/chemokine-receptor network could shed light on the cause and progression of pulmonary sarcoidosis and other inflammatory and autoimmune diseases and eventually lay the groundwork for therapeutic options.

2. Materials and Methods

2.1. Subjects. Patients were further subdivided according to the disease development as assessed by 2-year follow-up. BAL was performed according to a standard protocol [18] in 48 patients with pulmonary sarcoidosis (S) and 14 control subjects (C) of Czech origin. The diagnosis of sarcoidosis was determined in compliance with the criteria from the Statement on Sarcoidosis [19]. No patient received corticosteroid treatment before BAL. Patients were further subdivided according to the disease development as assessed by the 2-year follow-up: (i) patients with progressing disease (Prog, $n = 20$) and (ii) those where the regression was achieved (Reg, $n = 28$). The control group consisted of 14 subjects undergoing BAL as a part of clinical investigation for psychogenic cough, cough associated with gastroesophageal reflux disease and lung hypertension. For clinical and laboratory characteristics of enrolled patients and control subjects, see Table 1.

All patients gave their informed consent for the use of BAL, taken primarily for diagnostic evaluation, for the purpose of this study. The local ethical committee of Palacký University and Faculty Hospital, Olomouc, approved the study.

2.2. BAL Sample Processing, miRNA/mRNA Isolation, and Reverse Transcription. BAL cells were separated from the fluid by centrifugation and total RNA was isolated from unseparated BAL cells with mirVana miRNA kit (Ambion, Austin, USA); RNA quality and quantity were measured by 2100 Bioanalyzer using RNA 6000 Nano assays (Agilent Technologies, Palo Alto, USA). The reverse transcription of miRNA was performed with TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) using stem-loop RT primers, ensuring RT efficiency and specificity [20] according to the manufacturer's instructions. The reactions were incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. The resulting cDNA was stored at −20°C until use. The reverse transcription of mRNA was performed with Transcriptor First Strand cDNA Synthesis Kit with anchored dT primers (Roche Applied Science, Indianapolis, USA) according to the manufacturer's recommendation. The reactions were incubated for 10 min at 65°C, 60 min at 50°C, and 5 min at 85°C. The resulting cDNA was stored at −20°C until use.

2.3. Measurement of miRNA/mRNA Expression by Quantitative RT-PCR. The gene expression for each miRNA and mRNA in BAL cells was investigated by qRT-PCR using specific primers and probes (see Table S1 and Table S2, in Supplementary Material available online at http://dx.doi.org/10.1155/2015/121378). qRT-PCR for each individual miRNA was performed in a 20 μL reaction mixture that included 1.3 μL of diluted RT product, 1 μL of 20X TaqMan Individual microRNA assay, 10 μL of 2X TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), and 7.7 μL of nuclease-free water. qRT-PCR for each individual mRNA expression was performed as described previously [21]. All reactions were performed on RotorGene3000 system (Qiagen Inc., Valencia, CA, USA); the reaction steps were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The relative miRNA and mRNA expression levels were calculated by a second-derivative method (RotorGene Software 6.1.81, Corbett, Sydney, Australia); cDNA from human universal reference RNA (Stratagene, La Jolla, CA, USA) was used as a calibrator. A reference gene for miRNA analysis was endogenous control Mammalian U6 and for mRNA a reference gene *PSMB2* [21]. Changes in expression levels are presented as mean relative expression with 95% confidence interval (CI).

2.4. Selection of Candidate miRNAs and Identification of Binding Sites. The miRNA pathway analysis web server DIANA-mirPath v.3 (http://www.microrna.gr/miRPathv3/) [22] was used to nominate the candidate inflammation-related miRNAs based on their possible involvement in cytokine-chemokine interaction pathway. For the identification of binding sites between candidate miRNAs and mRNAs, we used the mirSystem [23] (Table S3, Supplementary Material). This web-based tool integrates the seven most often used target gene prediction algorithms: DIANA, miRanda, miRBridge, PicTar, PITA, rna22, and TargetScan. Moreover, it contains validated data of interactions between particular

Table 1: Clinical and laboratory data of enrolled patients with pulmonary sarcoidosis.

Characteristics	Sarcoidosis ($N = 48$)	Regression ($N = 28$)	Progression ($N = 20$)	Controls ($N = 14$)
Age, yrs	47.9 (29–72)	45.8 (29–70)	50.9 (32–72)	39.6 (19–63)
Sex (male/female)	23/25	12/16	11/9	9/5
Smoking (y/n/ex)	0/37/11	0/20/8	0/17/3	0/9/5
Pulmonary/pulmonary plus extrapulmonary involvement	35/13	18/10	17/3	—
CXR stages (I/II)	15/33	10/18	5/15	—
Löfgren's syndrome (y/n)	7/41	4/24	3/17	—
BALF differential count[†]				
% macrophages	75.6 ± 5.8 (40.0–94.0)	74.3 ± 6.6 (40.0–94.0)	77.5 ± 4.5 (60.5–87.4)	92.2 ± 13.5 (83.8–97.4)
% lymphocytes	21.6 ± 5.5 (4.6–49.0)	22.8 ± 6.2 (6.0–49.0)	19.9 ± 4.6 (4.6–36.0)	6.2 ± 2.3 (1.6–11.0)
% neutrophils	2.1 ± 1.6 (0.0–18.0)	2.3 ± 1.8 (0.0–18.0)	1.8 ± 1.2 (0.0–10.0)	1.5 ± 0.8 (0.3–6.0)
% eosinophils	1.3 ± 1.9 (0.0–17.3)	1.3 ± 1.6 (0.0–17.0)	1.4 ± 1.9 (0.0–17.3)	0.3 ± 0.1 (0.3–0.6)
% CD3+[#]	83.6 ± 6.4 (48.0–98.0)	83.8 ± 7.1 (48.0–96.0)	83.3 ± 5.7 (54.0–98.0)	74.1 ± 7.2 (40.0–92.0)
% CD4+[#]	64.9 ± 8.8 (23.0–92.0)	66.9 ± 8.9 (27.0–92.0)	62.0 ± 8.9 (23.0–86.0)	45.7 ± 7.3 (22.0–67.0)
% CD8+[#]	16.8 ± 5.7 (2.0–50.0)	14.4 ± 4.9 (2.0–42.0)	20.3 ± 6.4 (4.0–50.0)	27.4 ± 5.5 (14.0–51.0)
% CD19+[#]	1.03 ± 1.2 (0.0–14.0)	0.9 ± 1.3 (0.0–14.0)	1.2 ± 1.1 (0.0–8.0)	1.2 ± 0.6 (0.0–3.0)
BALF CD4+/CD8+ ratio	6.8 ± 3.6 (0.8–46.0)	7.9 ± 4.2 (1.1–46.0)	5.3 ± 2.6 (0.8–21.3)	2.0 ± 0.5 (0.5–3.7)

BALF: bronchoalveolar lavage fluid; *N*: number of patients; n: no; y: yes; ex: ex-smoker; CXR: chest X-ray; —: not relevant.
Data are presented as mean ± SD (minimum and maximum in parentheses).
[†]Data were not available for four patients.
[#]% of CD3, CD4, CD8, and CD19 refers to total lymphocyte counts.

miRNA and its target genes from TarBase and miRecords databases.

2.5. Clustering Using Kohonen Self-Organizing Neural Networks. Kohonen self-organizing neural network (self-organizing maps, SOM), a clustering tool, was applied to find clusters of input data that are very close to each other [24]. The input data (the whole data set including miRNA and mRNA expression of all studied molecules) for each sarcoidosis patient were transformed to vectors, which were recorded in the neural network. Neurons in the cortex were organized in 2D; only the adjacent neurons were interconnected. If the data clustered to neurons in SOM show the internal clustering structure corresponding to the analyzed subgroups (in our case progressing and regressing disease), the data set has high potential to contain relevant biological relationships.

2.6. miRNA/mRNA Correlation Analysis. Correlation matrices were computed to investigate the relationships between miRNA expression and mRNA expression of *T-bet* and members of sarcoidosis-associated cytokine/chemokine-receptor network represented by Spearman correlation coefficient. The correlation matrices were graphically presented using heat maps, where a hierarchical agglomerative clustering analysis was performed to show the relationships between groups of miRNA and mRNA in the form of clusters. The colour of each cell of the heat map corresponds to the value of Spearman correlation coefficient between given miRNA and corresponding mRNA.

2.7. Circos Diagrams for Visualization of miRNA/mRNA Relationships. To graphically represent the relationships between miRNAs, mRNAs, and *T-bet*, a Chord diagram generated in Circos system [25] (http://mkweb.bcgsc.ca/tableviewer/) was applied for (i) sarcoidosis patients and subgroups of patients with (ii) progressing sarcoidosis and (iii) regressing sarcoidosis. In the diagram, only significant correlations are depicted ($P < 0.05$). The intensity of correlations (w) between individual miRNA-mRNA pairs corresponds to absolute value of Spearman correlation coefficient (r_s), for which the mathematical transformation was performed to accentuate the differences in the intensities of correlations. For the transformation, the following formula was used:

$$w = 1 + \left[10 \times \left(r_s - t\right)\right]^2, \quad (1)$$

where t represents the threshold for the highest significant P value.

2.8. miRNA/mRNA/T-bet Network Analysis. For schematic representation of the relationships between miRNAs, *T-bet*, and cytokines/chemokines/receptors, we constructed weighted gene coexpression network [26]. Further, an algorithm based on analysis of the nearest neighbors between the studied molecules (represented as vertices) was applied [27]. The larger vertices (spheres) in the network have more nearest neighbors based on correlation analysis than smaller vertices. Only edges connecting the nearest neighbors (pairs with the highest correlations) were preserved. In other words, the size of the vertices (spheres) and connection among vertices show relationships between the investigated molecules in the network.

2.9. Statistical Analysis. Data analysis was performed using GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA, USA) and SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Differences in miRNA and mRNA levels between the cohorts were assessed by nonparametric Kruskal-Wallis one-way analysis-of-variance-by-ranks test; nonparametric Mann-Whitney test was utilized to determine significant differences between two groups. Spearman correlation coefficient and its corresponding P values were computed using R statistical software package (http://www.r-project.org/). A P value < 0.05 was considered significant.

3. Results

3.1. Selection of Studied miRNAs. Candidate miRNAs were selected based on their possible involvement in the regulation of inflammatory response, particularly cytokine/chemokine-receptor network. Using DIANA-mirPath v.3, we nominated 25 candidate miRNAs (*miR-let-7c, let-7d, miR-21, miR-24, miR-25*, *miR-92a*, *miR-125a*, *miR-126*, *miR-133a*, *miR-146a*, *miR-148a*, *miR-150*, *miR-155*, *miR-181a*, *miR-199a*, *miR-202*, *miR-204*, *miR-206*, *miR-212*, *miR-214*, *miR-222*, *miR-223*, *miR-302c*, *miR-424*, and *miR-503*) targeting cytokine/chemokine-receptor network (see Figure S1, Supplementary Material), involving many of the cytokines/chemokines and their receptors associated with sarcoidosis. A list of miRBase ID numbers and other details on studied miRNAs are stated in Table S1 (Supplementary Material); the miRNAs having (3′-UTR) "seed region" for binding to the studied mRNA are stated in Table S3 (Supplementary Material).

3.2. miRNA Expression Profiling

3.2.1. Analysis of miRNA Expressions in Sarcoidosis Patients and Control Subjects. In order to investigate the miRNA expression pattern, the expression levels of *miR-let-7c*, *let-7d*, *miR-21*, *miR-24*, *miR-25*, *miR-92a*, *miR-125a*, *miR-126*, *miR-133a*, *miR-146a*, *miR-148a*, *miR-150*, *miR-155*, *miR-181a*, *miR-199a*, *miR-202*, *miR-204*, *miR-206*, *miR-212*, *miR-214*, *miR-222*, *miR-223*, *miR-302c*, *miR-424*, and *miR-503* were determined in BAL cells obtained from sarcoidosis patients and control subjects.

When compared to control subjects, a higher number of *miR-150* ($P < 0.001$) and *miR-146a* ($P = 0.006$) (Table 2, Figure 2(a)) transcripts and lower *miR-202* ($P = 0.036$), *miR-204* ($P = 0.031$), and *miR-222* ($P = 0.012$) (Table 2, Figure 2(a)) expression were detected in sarcoidosis patients. No difference in expression levels of other studied miRNAs was observed between sarcoidosis and control subjects (Figure 1(a)).

3.2.2. Analysis of miRNA Expressions in Sarcoidosis Patients with Progressing/Regressing Disease. In order to investigate the miRNA expression pattern in sarcoidosis patients subdivided according to disease outcome after 2-year follow-up, the expression levels of a set of candidate miRNAs were determined in BAL cells from progressing and regressing sarcoidosis. To exclude the notion that variations in the levels of expression of transcripts might be related to changes in cell populations, we investigated the distribution of the absolute and relative number of BAL macrophages and lymphocytes and revealed no difference between subgroups of patients with progressing and regressing sarcoidosis ($P > 0.05$).

When comparing to regressing sarcoidosis, elevated expression of *miR-155* ($P = 0.017$) and *let-7c* ($P = 0.039$) (Table 2, Figure 2(b)) was detected in progressing disease. No difference in expression levels of other studied miRNAs was observed between patients with progressing and regressing sarcoidosis (Figure 1(a)).

3.3. mRNA Expression Profiling

3.3.1. Analysis of mRNA Expressions in Sarcoidosis Patients and Control Subjects. In order to investigate the mRNA expression pattern in sarcoidosis, the expression levels of CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL13, CCL19, CXCL2, CXCL3, CXCL9, CXCL10, CXCL11, CXCL12, CXCL16, CCR1, CCR2A, CCR2B, CCR5, CXCR3, CXCR4, CXCR6, CXCR7, IL2, IL2RA, IL2RB, IL15RA, IFNG, and *T-bet* were determined in BAL cells obtained from sarcoidosis patients and control subjects.

When comparing to control subjects, the expression levels of CC chemokines CCL3 ($P = 0.043$), CCL4 ($P = 0.034$), CCL5 ($P < 0.001$), and CCL8 ($P = 0.031$) and CXC chemokines CXCL9 ($P < 0.001$), CXCL10 ($P = 0.002$), and CXCL11 ($P = 0.008$) were found to be elevated in sarcoidosis patients (Table 3, Figure 1(b)). Among chemokine receptors, mRNA expressions of CCR2-var.A ($P = 0.018$), CCR5 ($P = 0.003$), CXCR3 ($P < 0.001$), and CXCR6 ($P < 0.001$) were elevated in sarcoidosis patients (Table 3, Figure 1(b)). Among cytokines/cytokine receptors, the expression levels of IL2 ($P < 0.001$), IL2RB ($P = 0.049$), IL15RA ($P = 0.048$), and IFNG ($P < 0.001$) were elevated in sarcoidosis patients (Table 3, Figure 1(b)). *T-bet* mRNA expression was also found to be elevated in sarcoidosis patients ($P = 0.006$) comparing to control subjects (Table 3, Figure 1(b)).

3.4. Self-Organizing Neural Networks for Sarcoidosis Patients. In order to assess the inner data structure in our data sets, we applied the Kohonen SOM. The unsupervised clustering analysis classified the sarcoidosis patients into the following clusters represented by neurons (hexagons): (1) green

Table 2: Comparison of miRNA expression profiles between sarcoidosis (S) patients, subdivided according to the outcome after 2-year follow-up (Reg: regression; Prog: progression), and control subjects (C). All the data are presented as Mean Expression Level (95% confidence interval). Results are expressed relative to levels of endogenous U6 miRNA. *N*: number of patients. Bold font indicates significant *P* value.

	S ($N = 48$)	Reg S ($N = 28$)	Prog S ($N = 20$)	C ($N = 14$)	*P* (Kruskal-Wallis)	*P* S versus C	*P* Reg versus Prog
miR-let-7c-5p	0.058 (0.051–0.066)	0.051 (0.042–0.060)	0.069 (0.056–0.082)	0.069 (0.052–0.087)	0.127	0.239	**0.039**
miR-let-7d-5p	0.136 (0.118–0.154)	0.124 (0.100–0.147)	0.153 (0.126–0.181)	0.155 (0.120–0.190)	0.250	0.256	0.103
miR-21-5p	2.022 (1.618–2.427)	1.676 (1.300–2.051)	2.508 (1.693–3.323)	2.023 (1.253–2.794)	0.585	0.833	0.161
miR-24-3p	0.522 (0.462–0.582)	0.488 (0.409–0.567)	0.570 (0.473–0.667)	0.571 (0.432–0.711)	0.432	0.405	0.174
miR-25-3p	0.110 (0.071–0.150)	0.117 (0.050–0.184)	0.101 (0.077–0.124)	0.089 (0.042–0.137)	0.485	0.434	0.191
miR-92a-3p	0.364 (0.312–0.416)	0.319 (0.255–0.384)	0.421 (0.336–0.506)	0.362 (0.272–0.452)	0.277	0.814	0.061
miR-125a-5p	0.044 (0.034–0.054)	0.046 (0.029–0.062)	0.042 (0.031–0.052)	0.058 (0.020–0.096)	0.982	0.814	0.770
miR-126-3p	0.054 (0.005–0.102)	0.077 (0.007–0.160)	0.021 (0.012–0.030)	0.057 (0.004–0.118)	0.992	0.860	0.810
miR-133a-3p	0.012 (0.010–0.015)	0.012 (0.008–0.015)	0.013 (0.009–0.017)	0.014 (0.010–0.018)	0.589	0.248	0.557
miR-146a-5p	0.304 (0.266–0.342)	0.294 (0.235–0.352)	0.319 (0.271–0.366)	0.196 (0.135–0.257)	**0.020**	**0.006**	0.331
miR-148a-3p	0.061 (0.050–0.072)	0.056 (0.042–0.069)	0.068 (0.049–0.087)	0.074 (0.050–0.097)	0.507	0.342	0.268
miR-150-5p	0.280 (0.219–0.340)	0.277 (0.199–0.355)	0.283 (0.179–0.386)	0.093 (0.061–0.125)	<**0.001**	<**0.001**	0.983
miR-155-5p	0.200 (0.170–0.231)	0.169 (0.138–0.200)	0.244 (0.187–0.300)	0.168 (0.126–0.210)	0.111	0.550	**0.017**
miR-181a-5p	0.106 (0.091–0.121)	0.102 (0.084–0.120)	0.112 (0.085–0.139)	0.122 (0.091–0.153)	0.650	0.263	0.630
miR-199a-5p	0.012 (0.010–0.014)	0.011 (0.008–0.014)	0.013 (0.009–0.016)	0.016 (0.007–0.024)	0.919	0.748	0.532
miR-202-3p	0.009 (0.008–0.011)	0.010 (0.007–0.013)	0.013 (0.006–0.020)	0.014 (0.009–0.019)	0.173	**0.036**	0.564
miR-204-5p	0.008 (0.006–0.010)	0.006 (0.005–0.007)	0.010 (0.005–0.015)	0.011 (0.007–0.015)	**0.030**	**0.031**	0.061
miR-206	0.026 (0.002–0.064)	0.039 (0.003–0.101)	0.010 (0.001–0.021)	0.005 (0.003–0.007)	0.999	0.928	0.963
miR-212-3p	0.010 (0.008–0.013)	0.010 (0.008–0.012)	0.011 (0.006–0.017)	0.012 (0.009–0.016)	0.490	0.158	0.705
miR-214-3p	0.009 (0.006–0.011)	0.008 (0.006–0.010)	0.010 (0.005–0.014)	0.010 (0.005–0.014)	0.968	0.866	0.621
miR-222-3p	0.038 (0.029–0.048)	0.059 (0.047–0.072)	0.065 (0.050–0.080)	0.062 (0.052–0.071)	0.059	**0.012**	0.668
miR-223-3p	0.808 (0.679–0.936)	0.815 (0.615–1.014)	0.798 (0.644–0.951)	0.901 (0.695–1.106)	0.692	0.270	0.826
miR-302c-3p	0.061 (0.048–0.074)	0.060 (0.041–0.079)	0.065 (0.046–0.083)	0.089 (0.034–0.143)	0.820	0.427	0.631
miR-424-5p	0.103 (0.002–0.289)	0.013 (0.002–0.025)	0.009 (0.006–0.012)	0.018 (0.005–0.031)	0.426	0.143	0.905
miR-503-5p	0.038 (0.030–0.046)	0.041 (0.030–0.052)	0.035 (0.023–0.046)	0.058 (0.022–0.094)	0.480	0.643	0.112

Table 3: Comparison of mRNA expression profiles of studied cytokines, chemokines, chemokine receptors, and *T-bet* between sarcoidosis (S) patients, subdivided according to the outcome after 2-year follow-up (Reg: regression; Prog: progression), and control subjects (C). All the data are presented as Mean Expression Level (95% confidence interval). Results are expressed relative to levels of a housekeeping gene PSMB2. *N*: number of patients. Bold font indicates significant *P* value.

	S ($N = 48$)	Reg S ($N = 28$)	Prog S ($N = 20$)	C ($N = 14$)	*P* (Kruskal-Wallis)	*P* C versus S	*P* Reg versus Prog
CCL2	0.058 (0.051–0.066)	0.051 (0.042–0.060)	0.069 (0.056–0.082)	0.069 (0.052–0.087)	0.950	0.602	0.875
CCL3	0.136 (0.118–0.154)	0.124 (0.100–0.147)	0.153 (0.126–0.181)	0.155 (0.120–0.190)	0.110	**0.043**	0.237
CCL4	2.022 (1.618–2.427)	1.676 (1.300–2.051)	2.508 (1.693–3.323)	2.023 (1.253–2.794)	0.157	**0.034**	0.746
CCL5	0.522 (0.462–0.582)	0.488 (0.409–0.567)	0.570 (0.473–0.667)	0.571 (0.432–0.711)	**0.002**	<**0.001**	0.834
CCL7	0.110 (0.071–0.150)	0.117 (0.050–0.184)	0.101 (0.077–0.124)	0.089 (0.042–0.137)	0.500	0.152	1.000
CCL8	0.364 (0.312–0.416)	0.319 (0.255–0.384)	0.421 (0.336–0.506)	0.362 (0.272–0.452)	0.147	**0.031**	1.000
CCL13	0.044 (0.034–0.054)	0.046 (0.029–0.062)	0.042 (0.031–0.052)	0.058 (0.020–0.096)	0.861	0.939	0.420
CCL19	0.054 (0.005–0.102)	0.077 (0.007–0.160)	0.021 (0.012–0.030)	0.057 (0.004–0.118)	0.477	0.216	0.403
CXCL2	0.012 (0.010–0.015)	0.012 (0.008–0.015)	0.013 (0.009–0.017)	0.014 (0.010–0.018)	0.429	0.414	0.161
CXCL3	0.304 (0.266–0.342)	0.294 (0.235–0.352)	0.319 (0.271–0.366)	0.196 (0.135–0.257)	0.069	0.608	**0.011**
CXCL9	0.061 (0.050–0.072)	0.056 (0.042–0.069)	0.068 (0.049–0.087)	0.074 (0.050–0.097)	**0.005**	<**0.001**	0.670
CXCL10	0.280 (0.219–0.340)	0.277 (0.199–0.355)	0.283 (0.179–0.386)	0.093 (0.061–0.125)	**0.009**	**0.002**	0.925
CXCL11	0.200 (0.170–0.231)	0.169 (0.138–0.200)	0.244 (0.187–0.300)	0.168 (0.126–0.210)	**0.045**	**0.008**	0.754
CXCL12	0.106 (0.091–0.121)	0.102 (0.084–0.120)	0.112 (0.085–0.139)	0.122 (0.091–0.153)	0.180	0.101	0.204
CXCL16	0.012 (0.010–0.014)	0.011 (0.008–0.014)	0.013 (0.009–0.016)	0.016 (0.007–0.024)	0.241	0.982	0.612
CCR1	0.009 (0.008–0.011)	0.010 (0.007–0.013)	0.013 (0.006–0.020)	0.014 (0.009–0.019)	0.289	0.277	0.112
CCR2-var.A	0.008 (0.006–0.010)	0.006 (0.005–0.007)	0.010 (0.005–0.015)	0.011 (0.007–0.015)	0.051	**0.018**	0.241
CCR2-var.B	0.026 (0.002–0.064)	0.039 (0.003–0.101)	0.010 (0.001–0.021)	0.005 (0.003–0.007)	0.779	0.444	0.587
CCR5	0.010 (0.008–0.013)	0.010 (0.008–0.012)	0.011 (0.006–0.017)	0.012 (0.009–0.016)	**0.016**	**0.003**	0.427
CXCR3	0.009 (0.006–0.011)	0.008 (0.006–0.010)	0.010 (0.005–0.014)	0.010 (0.005–0.014)	**0.002**	<**0.001**	0.691
CXCR4	0.038 (0.029–0.048)	0.059 (0.047–0.072)	0.065 (0.050–0.080)	0.062 (0.052–0.071)	0.295	0.152	0.271
CXCR6	0.808 (0.679–0.936)	0.815 (0.615–1.014)	0.798 (0.644–0.951)	0.901 (0.695–1.106)	<**0.001**	<**0.001**	0.616
CXCR7	0.061 (0.048–0.074)	0.060 (0.041–0.079)	0.065 (0.046–0.083)	0.089 (0.034–0.143)	0.667	0.392	0.417
IL2	0.103 (0.002–0.289)	0.013 (0.002–0.025)	0.009 (0.006–0.012)	0.018 (0.005–0.031)	<**0.001**	<**0.001**	0.730
IL2RA	0.038 (0.030–0.046)	0.041 (0.030–0.052)	0.035 (0.023–0.046)	0.058 (0.022–0.094)	0.210	0.051	0.730

Table 3: Continued.

	S ($N = 48$)	Reg S ($N = 28$)	Prog S ($N = 20$)	C ($N = 14$)	P (Kruskal-Wallis)	P C versus S	P Reg versus Prog
IL2RB	0.038 (0.029–0.048)	0.059 (0.047–0.072)	0.065 (0.050–0.080)	0.062 (0.052–0.071)	0.131	**0.049**	0.272
IL15RA	0.808 (0.679–0.936)	0.815 (0.615–1.014)	0.798 (0.644–0.951)	0.901 (0.695–1.106)	0.182	**0.048**	0.530
IFNG	0.061 (0.048–0.074)	0.060 (0.041–0.079)	0.065 (0.046–0.083)	0.089 (0.034–0.143)	**<0.001**	**<0.001**	0.565
T-bet	0.103 (0.002–0.289)	0.013 (0.002–0.025)	0.009 (0.006–0.012)	0.018 (0.005–0.031)	**0.031**	**0.006**	0.573

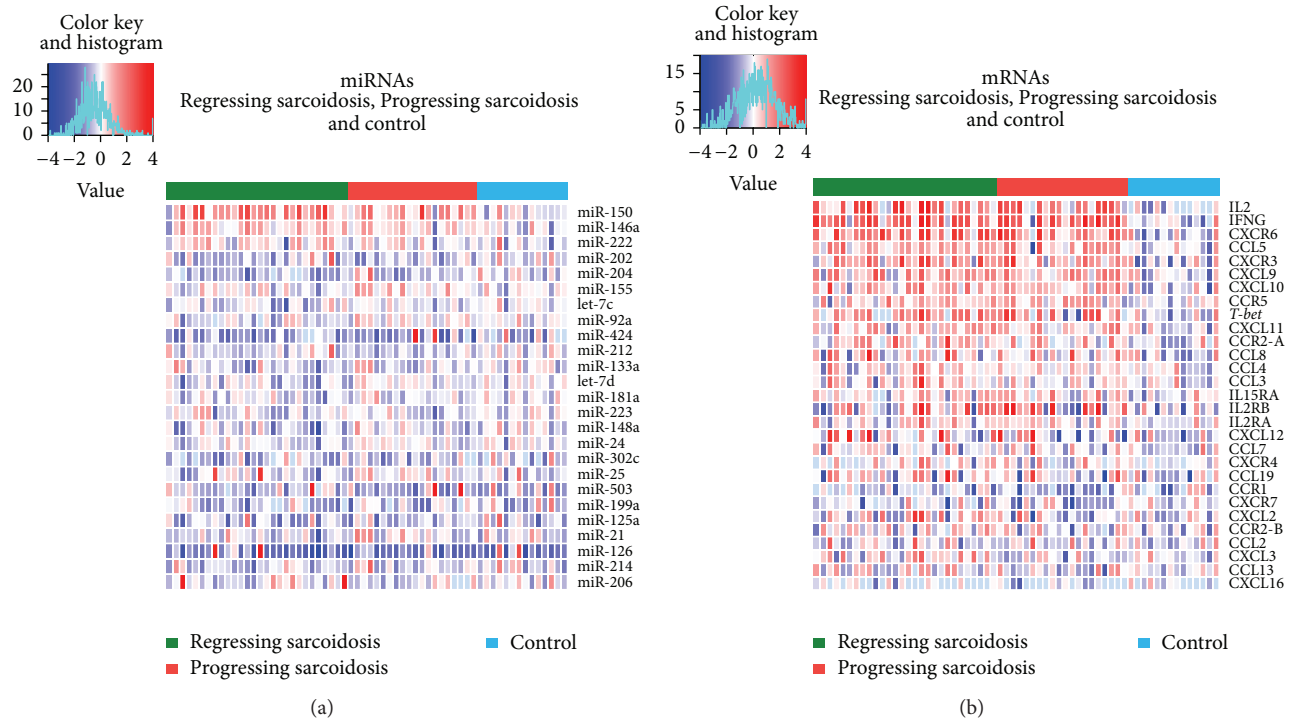


Figure 1: Hierarchical clustering of gene expression data. The rows represent individual (a) miRNAs and (b) mRNAs; the columns represent individual subject samples (1 column per sample) divided into three cohorts, such as sarcoidosis patients with progressing (red zone) and regressing (green zone) disease and control subjects (blue zone). The colour represents the gene expression level (blue: low expression, red: high expression); the expression levels are continuously mapped on the colour scale provided at the top of the figure. The analysis was performed using the R statistical software package (http://www.r-project.org/).

neurons including only patients with progression, (2) red neurons including only patients with regression; and (3) neurons including both classes visualized as interpolation between red and green colors (Figure 3).

This analysis supports the presence of inner structures in patient subgroups as assessed by disease outcome, thus supporting the fact that the subgroups differ in miRNA/mRNA profiles. Three clusters exactly matched patients with regressing sarcoidosis (25%) and one cluster patients with progressing disease (10.4%). Two clusters more or less correspond to patients with regressing sarcoidosis (27.1%), whereas one cluster more or less corresponds to patients with progressing disease (16.7%). On the other hand, two clusters of patients (20.8%) do not correspond to any class. Interestingly, the patients with regression formed more clusters than progressing patients, thus indicating that this subgroup is more heterogeneous regarding the expression profiles of studied miRNAs and mRNAs comparing to progressing disease.

3.5. Correlation Analysis of miRNAs, T-bet, and Cytokine/Chemokine-Receptor Network. In order to assess the relationships between studied miRNAs and mRNAs in BAL cells, we performed correlation analysis. To distinguish the degree of correlation between miRNA-mRNA pairs, we set up cut-off for Spearman correlation coefficient corresponding to significant P value ($P < 0.05$). Since no significant correlations between the studied molecules were detected

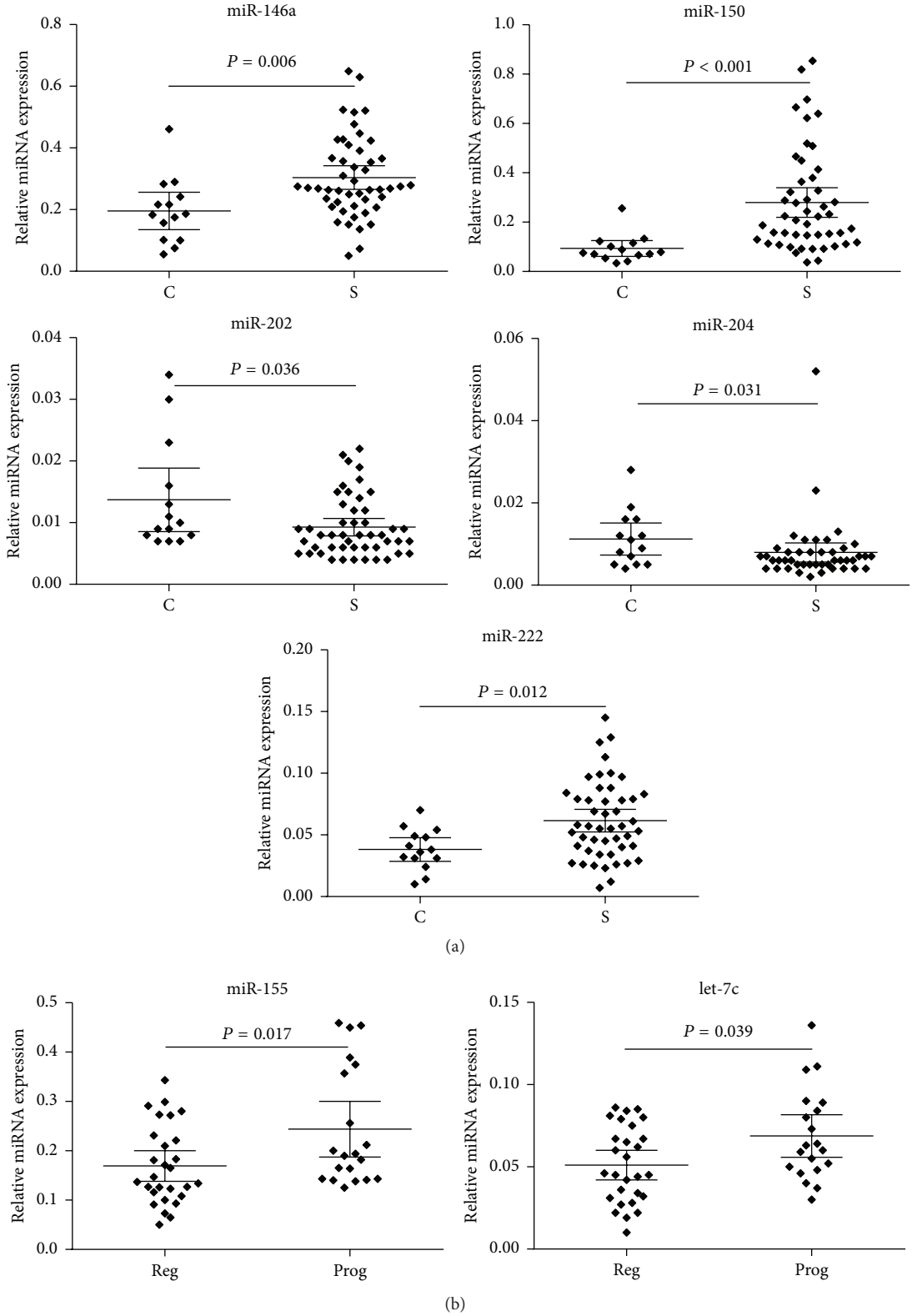


Figure 2: Distribution of relative miRNA expression (ratio target miRNA/reference endogenous U6 miRNA) of (a) five deregulated miRNAs between sarcoidosis patients (S) and control subjects (C) and (b) two deregulated miRNAs between patients with regressing (Reg) and progressing (Prog) sarcoidosis. Group means are indicated by horizontal bars; error bars indicate 95% CI.

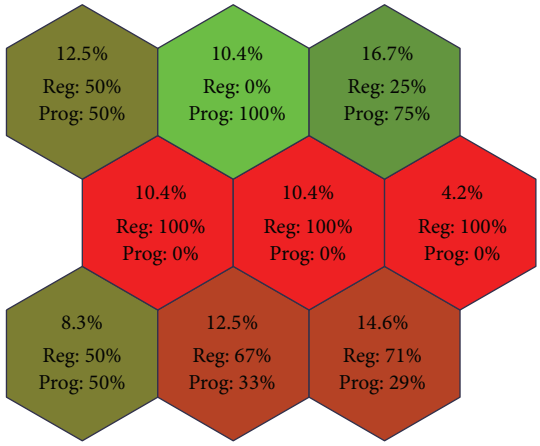


Figure 3: Kohonen self-organizing map (SOM) for a whole gene expression data set in BAL cells obtained from patients with sarcoidosis. Clustering shows the ratio of occurrence (given as % of patients/neuron) of two classes (progression and regression) in individual neurons (green neurons for patients with progression, red neurons for patients with regression; neurons classifying both classes are visualized as interpolation between red and green colors). The bold numbers indicate % of sarcoidosis patients clustered into particular neuron.

in control group, the Chord diagram for this group is not presented.

The most significant correlations in sarcoidosis as a whole were observed for the following miRNA-mRNA pairs: *miR-212*-CXCL10 ($P < 0.001$), *miR-24*-CXCR4 ($P < 0.001$), *miR-125a*-CCL7 ($P = 0.005$), *miR-146a*-CCL19 ($P = 0.003$), *miR-25*-CCL2 ($P = 0.002$), *miR-214*-CCL2 ($P = 0.008$), *miR-24*-CCL5 ($P = 0.010$), *miR-24*-CXCR3 ($P = 0.005$), *miR-21*-CXCR7 ($P = 0.003$), *miR-204*-IFNG ($P = 0.008$), *miR-148a*-CXCR4 ($P = 0.003$), and *miR-155*-CXCR4 ($P = 0.003$) (Figure 4(a)). Regarding *T-bet*, we observed correlations with CCL5 ($P < 0.001$), CXCR3 ($P < 0.001$), CXCR4 ($P < 0.001$), CXCR6 ($P < 0.001$), IL2 ($P < 0.001$), IL2RA ($P < 0.001$), IL2RB ($P < 0.001$), IL15RA ($P < 0.001$), IFNG ($P < 0.001$), CCR2B ($P = 0.004$), CXCL10 ($P = 0.021$), and CCR5 ($P = 0.021$) (Figure 4(a)).

In progressing disease, relationships for the following miRNA-mRNA pairs were observed: *miR-25*-CCL2 ($P < 0.001$), *miR-126*-CCL2 ($P < 0.001$), *miR-214*-CCL2 ($P < 0.001$), *miR-125a*-CCL7 ($P = 0.008$), *miR-126*-CCL7 ($P = 0.009$), *let-7d*-CXCR7 ($P = 0.001$), and *miR-126*-CXCR7 ($P = 0.009$) (Figure 4(b)). *T-bet* correlated with CCL5 ($P < 0.001$), CXCR3 ($P < 0.001$), CXCR4 ($P < 0.001$), IL2RB ($P < 0.001$), IL15RA ($P < 0.001$), IFNG ($P < 0.001$), CXCR6 ($P = 0.005$), CXCL10 ($P = 0.016$), CCR2B ($P = 0.029$), IL2 ($P = 0.044$), and IL2RA ($P = 0.037$) (Figure 4(b)).

In regressing sarcoidosis, we detected correlations among the following miRNA-mRNA pairs: *miR-146a*-CCL19 ($P < 0.001$), *let-7c*-CCL19 ($P = 0.007$), *miR-202*-CXCL10 ($P = 0.010$), *miR-212*-CXCL10 ($P = 0.002$), *miR-92a*-CXCL12 ($P = 0.008$), *miR-148a*-CXCR4 ($P = 0.002$), *miR-24*-IL2RB ($P = 0.006$), and *miR-25*-IL2RB ($P = 0.006$) (Figure 4(c)). Moreover, *T-bet* correlated with CXCR3 ($P < 0.001$), IL2 ($P < 0.001$), IL2RA ($P < 0.001$), IL2RB ($P < 0.001$), IL15RA ($P < 0.001$), IFNG ($P < 0.001$), CCL13 ($P = 0.004$), CXCL2 ($P = 0.004$), CXCR6 ($P = 0.007$), CXCL11 ($P = 0.013$), CCL5 ($P = 0.018$), CCR2A ($P = 0.033$), CCR2B ($P = 0.023$), and CXCR4 ($P = 0.017$) (Figure 4(c)).

In sarcoidosis patients, certain genes correlated with both *T-bet* and miRNAs simultaneously (Figure 4(d)). Correlation with both *T-bet* and miRNAs was observed for CCL5, CXCL10, CXCR3, CXCR4, CXCR6, IL2, IL15RA, and IFNG. In progressing sarcoidosis, correlation with both *T-bet* and miRNAs was observed for CXCR3 and IL2RB (Figure 4(b), Figure S2, Supplementary Material), whereas in regressing sarcoidosis *T-bet* correlated with CCL5, CCL13, CXCL11, CCR2A, CCR2B, CXCR3, CXCR4, IL2, IL2RB, IL15RA, and IFNG (Figure 4(c), Figure S2, Supplementary Material).

3.6. Weighted Gene Coexpression Network Analysis for miRNA-mRNA Relationships. In order to better characterize the relationships between studied miRNAs, mRNAs, and *T-bet*, we performed weighted gene coexpression network analysis.

The close relationships in sarcoidosis as a whole were found between *T-bet* and IL2RB, IL15RA, and CXCR3, which was related to CXCR6 and IFNG (Figure 5(a)). IFNG related to IL2 and CCL5 and also to *miR-204*. We observed relationships also between *miR-212*-CXCL10 and *miR-21*-CCR5. Considering miRNAs, we found relationships between *let-7d*, *miR-155*, *miR-24*, and *miR-25* and between *miR-202*, *miR-212*, *miR-424*, and *miR-503* (Figure 5(a)).

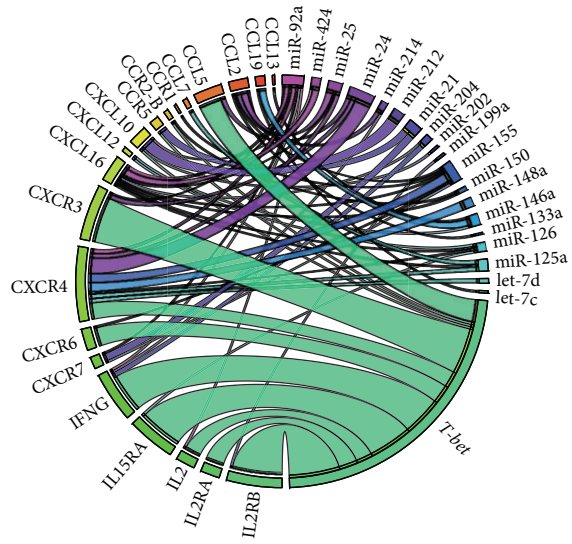
In progressing sarcoidosis, *T-bet* related to CCL5, IL2RB, IL15RA, and IFNG (Figure 5(b)). Relationships were observed also between *miR-212* and IFNG-induced chemokines CXCL9 and CXCL11. In addition, CXCL11 related to *miR-204*. Relationships were observed also between *miR-424* and CCL4 and CXCL10, whereas *miR-148a* related to CCR2A. Among miRNAs, relationships were found between *miR-21*, *miR-25*, *miR-148a*, *miR-92a*, and *let-7d* and between *miR-146a*, *miR-222*, and *let-7c* (Figure 5(b)).

In regressing sarcoidosis, relationships were observed between *T-bet* and IL2RB, IL15RA, CXCR3, IL2, and IFNG (Figure 5(c)). We observed relationships also between *miR-212* and CXCL10 and between *miR-503* and CCR2A. Among miRNAs, relationships were detected between *let-7d*, *miR-148a*, *miR-24*, and *miR-25*, between *miR-146a*, *miR-150*, *miR-222*, and *let-7c*, and between *miR-212*, *miR-202*, and *miR-503* (Figure 5(c)).

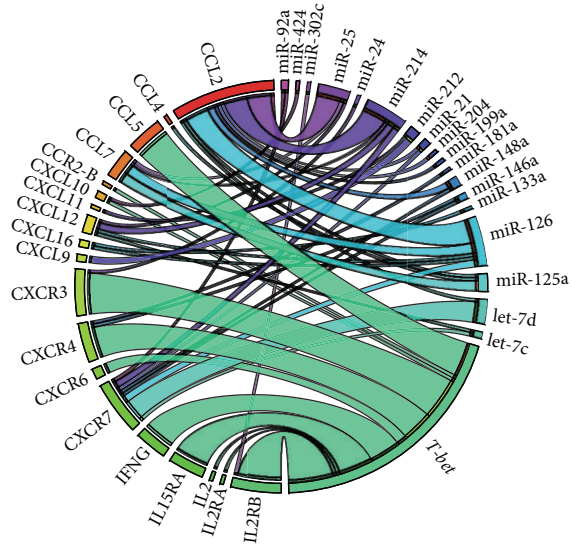
Regarding control group, the investigated molecules were not closely related to each other (small vertices) and their relationship pattern differed from sarcoidosis-associated pattern (Figure 5(d)).

4. Discussion

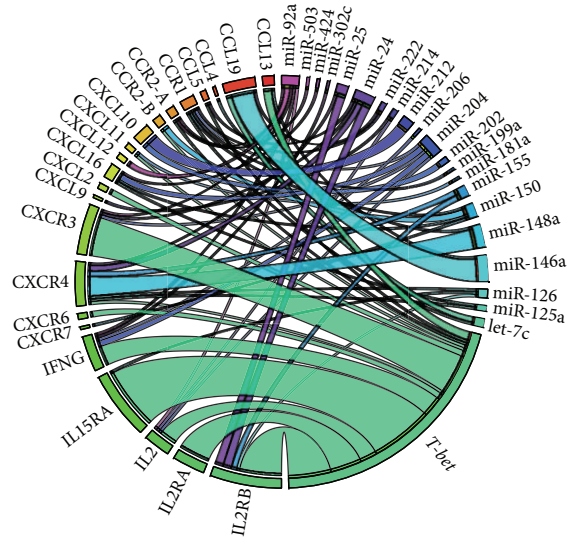
This is the first study investigating the suggested contribution of both transcriptional and posttranscriptional control to deregulated cytokine/chemokine-receptor network in pulmonary sarcoidosis. Our correlation network analysis revealed that both inflammatory-related microRNAs



(a) Sarcoidosis

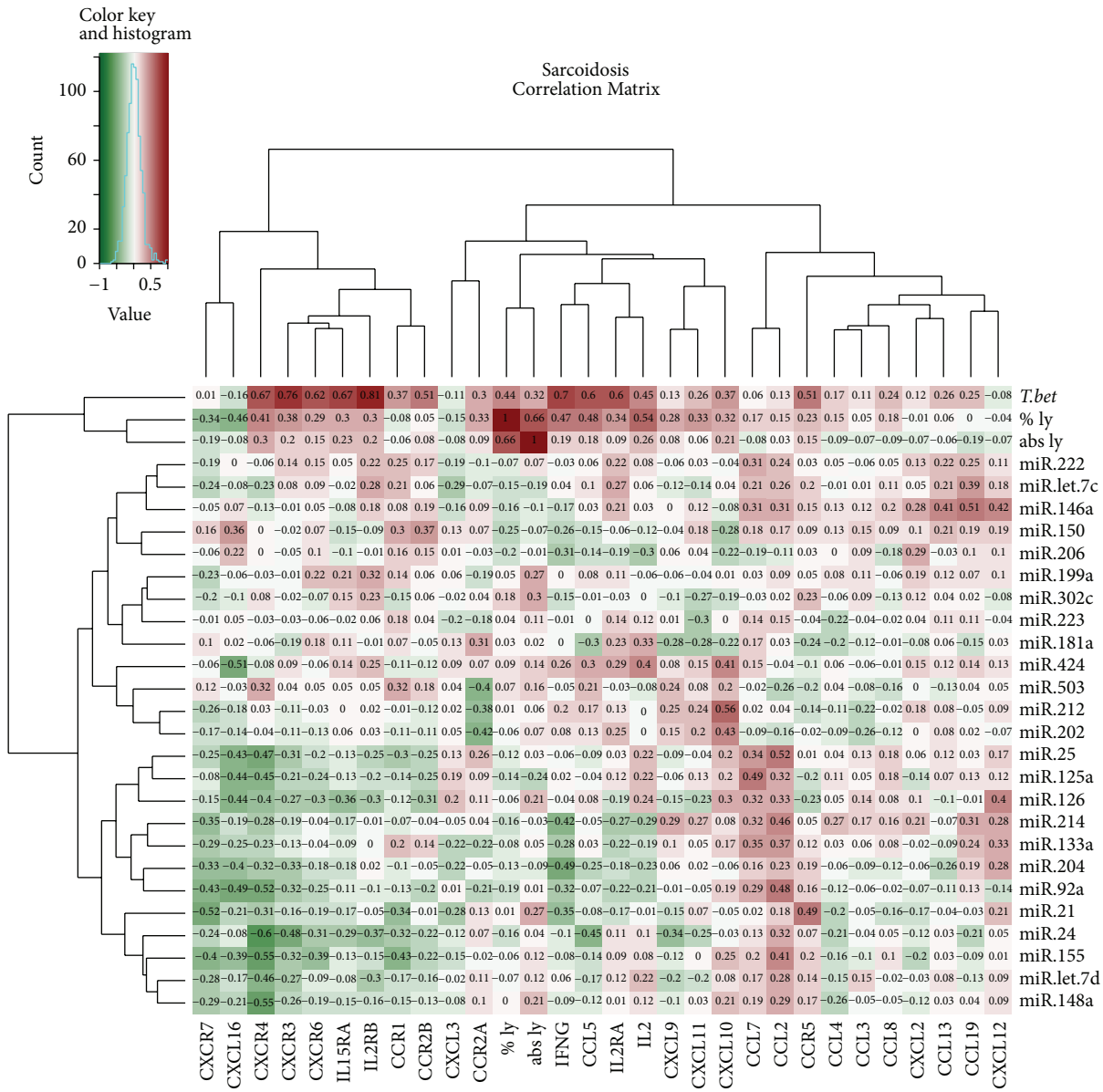


(b) Progressing sarcoidosis



(c) Regressing sarcoidosis

Figure 4: Continued.



(d)

Figure 4: miRNA-mRNA-*T-bet* correlations in BAL cells obtained from (a) sarcoidosis patients and their subgroups with (b) progressing and (c) regressing disease are represented using Chord diagrams (circular graphs). In the Chord diagram, the intensity of the band corresponds to the significance of the correlation between particular miRNA-mRNA pair and *T-bet*-mRNA pair as assessed using Spearman's rank correlation; only significant correlations ($P < 0.05$) are visualized. (d) A hierarchical agglomerative clustering analysis presented using heat map for sarcoidosis as a whole. The colour of each cell of the heat map corresponds to value of Spearman correlation coefficient between given miRNA-mRNA pairs. % ly: % of lymphocytes in BAL fluid; abs ly: absolute number of lymphocytes/1 mL BAL fluid.

and a key Th1-transcription factor *T-bet* may contribute to deregulated cytokine/chemokine-receptor network in sarcoid BAL cells, whereas contributions of transcriptional and posttranscriptional regulation differ between progressing and regressing disease as assessed by 2-year follow-up. We also for the first time investigated the gene profile of inflammation-related miRNAs in BAL cells and revealed altered miRNA pattern in BAL cells obtained from patients with sarcoidosis comparing to control subjects and also from patients with progressing versus regressing disease.

In sarcoidosis pathogenesis, the crucial role is attributed to inflammatory cytokines and chemokines, which direct circulating leukocytes to the sites of inflammation and control leukocyte activation and cytokine production, angiogenesis, and Th cell polarization [4, 28, 29]. In view of the crucial role of cytokines, chemokines, and their receptors in the pathogenesis of sarcoidosis, a fundamental question arises: how the cytokine/chemokine-receptor network is regulated. There is first evidence that cytokines and chemokines are regulated by both transcriptional and posttranscriptional mechanisms

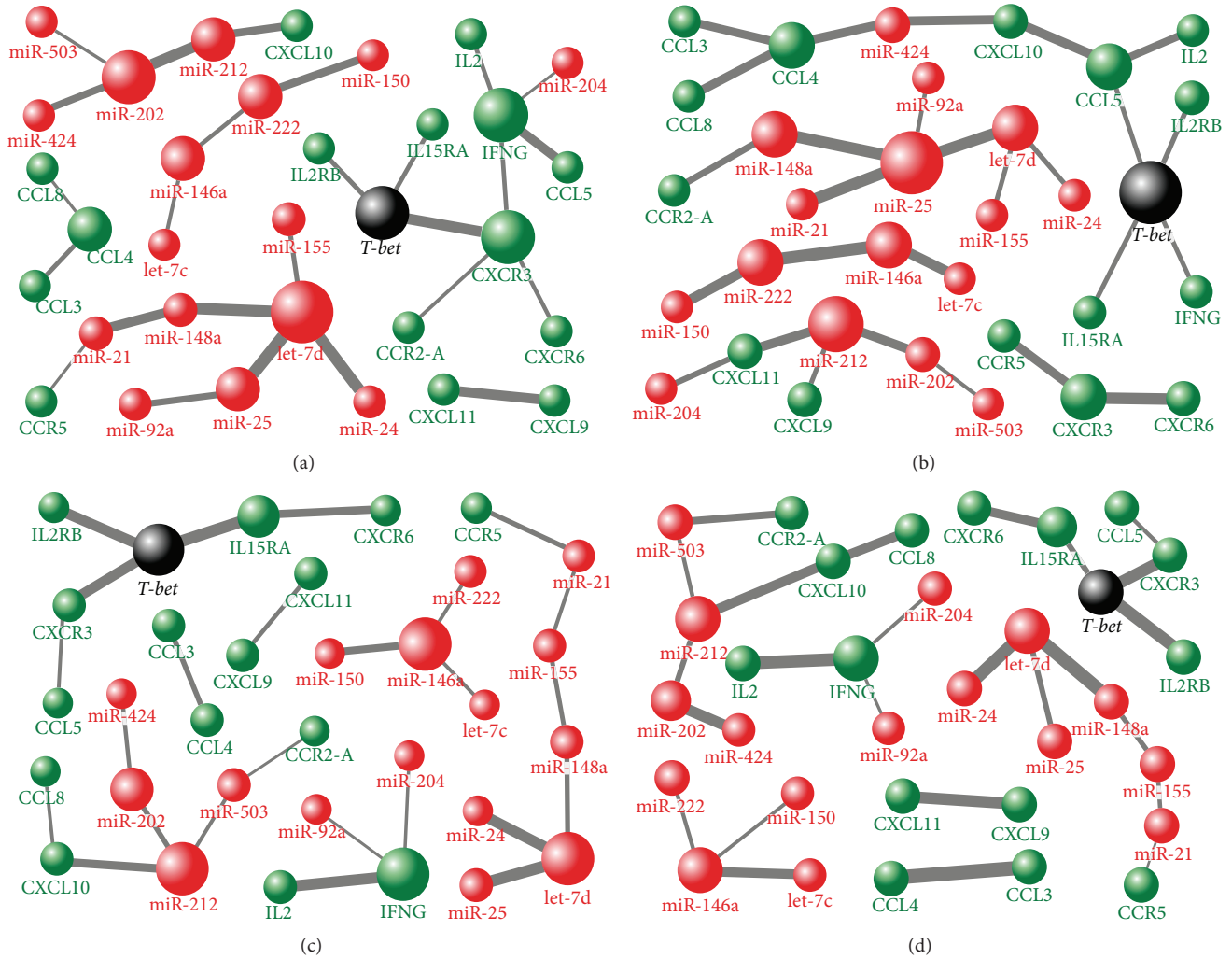


FIGURE 5: A weighted gene coexpression network analysis of miRNA-mRNA-*T-bet* relationships in BAL cells obtained from (a) sarcoidosis patients and their subgroups with (b) progressing and (c) regressing disease and from (d) control subjects. The studied molecules are represented as vertices (spheres), where larger vertices in the network have more nearest neighbors than smaller ones. Only edges connecting the nearest neighbors (pairs with the highest correlations) were preserved.

allowing for rapid and transient production in response to a variety of stimuli [30, 31]. In line with this observation, we hypothesized that the regulation of cytokine/chemokine-receptor network in BAL cells also involves coordinated participation of multiple regulatory systems, including miRNAs and a key sarcoidosis-associated Th1-transcription factor *T-bet*.

However, miRNAs pattern in BAL cells has not been investigated yet. We therefore investigated for the first time expression pattern of inflammation-related miRNAs in BAL cells and revealed downregulated *miR-202* and *miR-204* and upregulated *miR-146a*, *miR-150*, and *miR-222* expression in sarcoidosis comparing to controls. When comparing progressing to regressing sarcoidosis, a higher number of *miR-155* and *let-7c* transcripts were detected in progressing sarcoidosis. All detected miRNAs associated with sarcoidosis were already associated with regulation of inflammatory response. Inflammatory *miR-155* and *miR-146a* were found to be upregulated by NF-κB [32, 33], *miR-150* involved in pulmonary inflammation [34], *miR-202* in rheumatoid arthritis [35], and *miR-204* in the production of inflammatory mediators and apoptosis [36]. *MiR-222* contributed to inflammation-mediated neovessel formation [37], and *let-7c* regulated allergic airway inflammation [38]. Moreover, *miR-204* was also found to be deregulated in sarcoid lung tissues reported by Crouser et al. [9]. Interestingly, when comparing the miRNA profile in sarcoid BAL cells obtained in this study with profile reported in sarcoid lung tissues [9], we obtained different pattern of top-deregulated miRNAs (*miR-92a, miR-125a, miR-126, miR-181a, miR-206*, and *miR-302*) found in sarcoid lung tissues, thus supporting the fact that miRNA expression signatures vary depending on the tissues and cell types examined.

However, it is still unclear how miRNAs regulate cytokines, because 3′-untranslated region (3′-UTR) of most cytokines lacks critical "seed region" for binding to mRNA [39]. Current studies showed that cytokine and other immune genes may be regulated by other elements (e.g., ARE machinery components) [40] or through highly biologically functional "nonseed" miRNA target sites, containing single

mismatches, GU wobbles, insertions, or deletions in the seed-match regions [41–43]. These "nonseed" miRNA target sites represent up to 50% of all miRNA-mRNA interactions and remain uncovered, despite their functional significance, using current target prediction algorithms [39]. Importantly, miRNA regulation may not be directly at the cytokine/chemokine or receptor level but rather at upstream or downstream steps in the pathway. These facts may explain our observation of correlation between numerous miRNAs and cytokine expression (e.g., CXCR4 and *miR-24*), where no binding site was discovered by prediction algorithms as well as numerous positive correlations between miRNAs and cytokine and chemokine expression (e.g., CCL2 and *miR-214*) as reported also in other studies [44, 45]. Moreover, the unique role of miRNAs in inflammation is that the miRNAs modulate the expression of target genes to an optimum level rather than participating in on/off decisions [40].

Besides posttranscriptional regulation by miRNAs, also transcriptional control by transcription factors may contribute to deregulated cytokine/chemokine-receptor network in pulmonary sarcoidosis. We recently reported crucial role for a Th1-transcription factor *T-bet*, which has emerged as key regulator of IFNG and chemokine receptor CXCR3 and other immune genes in sarcoid inflammation [12]. In the present study, we searched for evidence of possible participation of transcriptional and posttranscriptional regulation by miRNAs and *T-bet* in controlling the inflammation in sarcoid BAL cells. Our correlation analysis revealed relationship of *T-bet* and sarcoid inflammation, namely, its association with key sarcoidosis-associated cytokine IFNG, and receptors IL2RB, IL15RA, CXCR3, and CXCR6, regardless of the disease outcome.

Importantly, the relationships between *T-bet* and particular miRNAs regarding the chemokine network changed between subgroups of patients, who differed in disease outcome after 2-year follow-up. In progressing sarcoidosis, *T-bet* correlated with CCL5, CXCR4, and CXCR7. We also observed positive correlations between numerous miRNAs and expression of CCL2, CCL7, and CXCL12 and negative correlation with CCL5, CXCR3, CXCR4, and CXCR7 expression in progressing disease. In regressing disease, *T-bet* showed less pronounced correlation with IL2 and receptors CXCR4, CXCR7, and IL2RA than in progression. Also miRNA pattern changed; in regression, the correlation analysis revealed positive correlation between *miR-146a* and CCL19 as well as *miR-148a* and CXCR4. Our analysis therefore nominated CCL2 and CCL5, both chemoattractants of mononuclear and mast cells [46], as well as CCL19, a chemokine implicated in T-lymphocyte recruitment [47], as key candidate chemokines associated with prognosis of pulmonary sarcoidosis. Similarly, CXCR4 and CXCR7 and their ligand, CXCL12, playing a role in regulation of leukocyte mobilization and trafficking [48, 49], deserve further investigation.

To better characterize the network of inflammation-related miRNAs, *T-bet*, and cytokine/chemokine-receptor network in sarcoid BAL cells, we performed weighted correlation network analysis. This analysis further supported the crucial relationships between *T-bet*, CCL5, IFNG, IL2RB, and IL15RA, together with *let-7d* and *miR-202* in progressing sarcoidosis. In regressing disease, *T-bet* was more closely related to CXCR3 and less to IFNG and CCL5 than in progressing disease. Regarding miRNAs in regressing disease, *miR-212*, *miR-146a*, and *let-7d* were highlighted. Given the complexity of miRNA-mRNA-transcription factor network, the understanding of the contribution of network members and regulatory mechanisms is intricate and requires further studies which may reveal the functional outcome of the interactions between the associated molecules.

Our study has several limitations. First, this exploratory study on miRNA and mRNA expression pattern and their relationships was performed in unseparated BAL cells. To obtain complete picture of the regulatory processes ongoing in sarcoid inflammation, this study should continue by analysing the miRNA/mRNA expressions in distinct cell subpopulations. We are also aware that our set of inflammation-related miRNAs and chemokine/receptor genes, selected based on their involvement in the pathogenesis of sarcoidosis, does not cover the whole potential network of molecules/pathways contributing to this disease. Further, we did not perform the functional studies needed to confirm the biological relevance of the obtained relationships between *T-bet*, miRNAs, and chemokine network. However, we believe that our study may contribute to nomination of interesting relationships for future studies of regulatory mechanisms involved in the pathogenesis of pulmonary sarcoidosis and its phenotypes.

5. Conclusions

Our correlation network analysis implies both microRNAs and Th1-transcription factor *T-bet* in the regulation of cytokine/chemokine-receptor network in BAL cells in sarcoidosis. Future functional studies are however needed to confirm the biological relevance of the obtained relationships. The correlation analysis showed more pronounced regulatory capability of *T-bet* to sarcoidosis-associated chemokine receptors and cytokines than miRNAs, which function rather as "fine-tuners" of cytokine/chemokine gene expression. Moreover, we reported altered miRNA pattern in BAL cells obtained from sarcoidosis patients as well as from subgroups of patients with progressing and regressing sarcoidosis as assessed by 2-year follow-up. The knowledge and understanding of the regulatory mechanisms underlying the abnormal inflammatory response in sarcoid lungs could shed light on the cause and progression of sarcoidosis and many inflammatory and autoimmune diseases.

Abbreviations of Investigated Molecules

CCL2/MCP-1:	Chemokine (C-C motif) ligand 2/monocyte chemoattractant protein-1
CCL3/MIP-1A:	Chemokine (C-C motif) ligand 3/macrophage inflammatory protein-1 alpha
CCL4/MIP-1B:	Chemokine (C-C motif) ligand 4/macrophage inflammatory protein-1 beta

CCL5/RANTES:	Chemokine (C-C motif) ligand 5/regulated upon activation, normally T-expressed, and presumably secreted
CCL7/MCP-3:	Chemokine (C-C motif) ligand 7/monocyte chemoattractant protein-3
CCL8/MCP-2:	Chemokine (C-C motif) ligand 8/monocyte chemoattractant protein-2
CCL13/MCP-4:	Chemokine (C-C motif) ligand 13/monocyte chemoattractant protein-4
CCL19/MIP-3B:	Chemokine (C-C motif) ligand 19/macrophage inflammatory protein-3 beta
CXCL2/MIP-2A:	Chemokine (C-X-C motif) ligand 2/macrophage inflammatory protein-2 alpha
CXCL3/MIP-2B:	Chemokine (C-X-C motif) ligand 3/macrophage inflammatory protein-2 beta
CXCL9/MIG:	Chemokine (C-X-C motif) ligand 9/monokine induced by gamma interferon
CXCL10/IP-10:	Chemokine (C-X-C motif) ligand 10/interferon gamma-induced protein-10
CXCL11/I-TAC:	Chemokine (C-X-C motif) ligand 11/interferon-inducible T-cell alpha chemoattractant
CXCL12/SDF-1:	Chemokine (C-X-C motif) ligand 12/stromal cell-derived factor-1
CXCL16/SR-PSOX:	Chemokine (C-X-C motif) ligand 16/scavenger receptor for phosphatidylserine and oxidized low density lipoprotein
CCR1:	Chemokine (C-C motif) receptor 1
CCR2A:	Chemokine (C-C motif) receptor 2, transcript variant A
CCR2B:	Chemokine (C-C motif) receptor 2, transcript variant B
CCR5:	Chemokine (C-C motif) receptor 5
CXCR3:	Chemokine (C-X-C motif) receptor 3
CXCR4:	Chemokine (C-X-C motif) receptor 4
CXCR6:	Chemokine (C-X-C motif) receptor 6
CXCR7:	Chemokine (C-X-C motif) receptor 7
IL2:	Interleukin 2
IL2RA:	Interleukin 2 receptor, alpha
IL2RB:	Interleukin 2 receptor, beta
IL15RA:	Interleukin 15 receptor, alpha
IFNG:	Interferon gamma
PSMB2:	Proteasome (prosome, macropain) subunit, beta type, 2
T-bet:	T-box 21, T-cell-specific T-box transcription factor.

Conflict of Interests

This work has not been supported by commercial sources and the authors are not aware of any potential conflict of interests.

Authors' Contribution

Tereza Dyskova and Regina Fillerova contributed equally to this study.

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Research Article

Altered Innate Immune Responses in Neutrophils from Patients with Well- and Suboptimally Controlled Asthma

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Background. Respiratory infections are a major cause of asthma exacerbations where neutrophilic inflammation dominates and is associated with steroid refractory asthma. Structural airway cells in asthma differ from nonasthmatics; however it is unknown if neutrophils differ. We investigated neutrophil immune responses in patients who have good (A_{Good}) and suboptimal (A_{Subopt}) asthma symptom control. *Methods*. Peripheral blood neutrophils from A_{Good} (ACQ < 0.75, $n = 11$), A_{Subopt} (ACQ > 0.75, $n = 7$), and healthy controls (HC) ($n = 9$) were stimulated with bacterial (LPS (1 μg/mL), fMLF (100 nM)), and viral (imiquimod (3 μg/mL), R848 (1.5 μg/mL), and poly I:C (10 μg/mL)) surrogates or live rhinovirus (RV) 16 (MOI1). Cell-free supernatant was collected after 1 h for neutrophil elastase (NE) and matrix metalloproteinase- (MMP-) 9 measurements or after 24 h for CXCL8 release. *Results*. Constitutive NE was enhanced in A_{Good} neutrophils compared to HC. fMLF stimulated neutrophils from A_{Subopt} but not A_{Good} produced 50% of HC levels. fMLF induced MMP-9 was impaired in A_{Subopt} and A_{Good} compared to HC. fMLF stimulated CXCL8 but not MMP-9 was positively correlated with FEV_1 and FEV_1/FVC. A_{Subopt} and A_{Good} responded similarly to other stimuli. *Conclusions*. Circulating neutrophils are different in asthma; however, this is likely to be related to airflow limitation rather than asthma control.

1. Introduction

Mainstay therapy for asthma is a combination of a long-acting β_2 agonist to relax smooth muscle in the airways and a corticosteroid to reduce inflammation in the lungs [1]. However, even at high doses of these medications some patients remain unresponsive. These patients with uncontrolled or difficult to treat asthma often have a neutrophilic phenotype [2, 3]; however a group of patients with a steroid refractory eosinophilic phenotype also exist [4, 5]. These patients not only are unable to obtain symptom relief but also suffer from more frequent and severe exacerbations [6, 7].

Asthma exacerbations may be triggered by a number of provokers of which the most common are respiratory infections. Viral infections have been extensively studied and rhinovirus (RV) is the most commonly detected virus in exacerbating adults [8]. Bacterial infections are not as rigorously examined but appear to also be clinically significant [9]. Respiratory infections pose a high threat to patients suffering from uncontrolled asthma as they can trigger exacerbations that are often severe and leave the patient hospitalised with limited treatment options [10, 11].

Neutrophils are the most abundant immune cell in the body; their main effector role is to control infections. CXCL8 is a potent neutrophil chemoattractant, neutrophil elastase (NE) has potent antimicrobial properties, and matrix metalloproteinase- (MMP-) 9 is important in activating antimicrobial peptides, all of which are released by neutrophils.

Table 1: Patient characteristics of healthy controls and asthmatics.

	Healthy controls	Asthma, well-controlled	Asthma, suboptimally controlled
N	9	11	7
Age years, mean (±SEM)	60.22 (±5.12)	62.64 (±3.97)	62.57 (±4.02)
Gender (M/F)	6/3	7/4	5/2
ACQ, mean (±SEM)	0 (±0)	0.32 (±0.08)*	1.33 (±0.17)****/####
FEV_1% pred. (±SEM)	92.78 (±5.98)	80.55 (±7.71)	70.29 (±8.09)
FEV_1/FVC% pred. (±SEM)	101.8 (±1.65)	88.36 (±4.51)*	77.43 (±5.27)**
Short-acting β_2 agonist use (Y/N)	0/9	9/0	7/0

* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ compared to healthy controls.
$p < 0.0001$ compared to patients with asthma with good symptom control.
ACQ: asthma control questionnaire.
FEV_1: forced expiratory volume in 1 second.
FVC: forced vital capacity.

Transient neutrophilia and neutrophilic inflammation are a normal phase of the immune response to pathogens [12]; however, chronic airway inflammation occurs in stable asthma [2, 13, 14]. In a study of 205 patients, multivariate linear regression has shown no association of airway neutrophilia with corticosteroid use [15], and airway neutrophilia occurs in asthmatic patients who are corticosteroid naïve [16]. In addition, neutrophil numbers [2] and neutrophil inflammatory mediators such as CXCL8 [17], NE [18], and MMP-9 [19] are elevated in the airways of patients with severe asthma and these levels correlate with disease severity [2, 20].

It has been shown that structural cells, such as epithelial and smooth muscle cells, in the asthmatic airway are different compared to nonasthmatic cells in both morphology and function [21–24]. It is believed that these functional abnormalities drive other changes in the airways which give rise to the hallmarks of asthma. We previously found that circulating neutrophils from patients with asthma are altered in their response to the viral mimetic, R848 by producing elevated levels of CXCL8 [25], and expression quantitative trait loci mapping in neutrophils has found immune dysfunction trait associated variants [26]. However, to date it has not been investigated if neutrophil functions differ in patients with suboptimal symptom control despite taking moderate to high dose steroid therapy. Neutrophil dysfunction may occur in these patients which would, in part, account for the greater inflammatory mediator load in this group of patients.

Neutrophils are produced in the bone marrow and have a relatively short life span [27, 28]. Given our previous finding of different responses of lung versus circulating neutrophils [29], to ascertain if neutrophils are already different prior to entering the lung in well- and suboptimally controlled asthma we compared the response to both bacterial and viral mimetics in circulating neutrophils to avoid any potential confounding effects of the lung inflammatory environment. We hypothesised that neutrophils from patients with suboptimally controlled asthma have a defective innate immunity which may predispose to pathogen-induced exacerbations.

2. Materials and Methods

2.1. Volunteer Recruitment. The project was approved by the Human Research Ethics Committee, The University of Sydney, prior to commencement. Volunteers with doctor diagnosed asthma, stable disease, and no reported symptoms of respiratory infection were recruited into the study. Healthy control volunteers were also recruited. Participants were required to be over 18 years of age and be fluent in English. Exclusion criteria included if they were pregnant, were known to faint during venipuncture procedures, or had a blood borne infection or condition. All volunteers provided written informed consent and were asked to complete a standardised questionnaire regarding age, gender, asthma symptoms, asthma medication use, and smoking history. Patients also completed baseline spirometry for forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC). Participants with asthma were asked to withhold their short-acting β_2 agonists for a minimum of 6 hours and 24 hours for long-acting β_2 agonists and inhaled corticosteroids (ICS).

2.2. Categorisation of Asthmatics. Participants with asthma were stratified based on their asthma control questionnaire (ACQ) score [30]. A cut point of ACQ < 0.75 was used to identify well-controlled asthma [30]. Participants with suboptimal asthma control (ACQ ≥ 0.75) also had evidence of variable airflow limitation (PD_{15} < 15 mL hypertonic saline or standard challenge agent, change in postbronchodilator FEV_1 > 12% of 200 mL, >12% peak flow variability over at least 1 week, or FEV_1 variability >12% of two measurements within two months of each other) and were taking a minimum of GINA step 3 maintenance combination therapy. Patient information is provided in Tables 1 and 2.

2.3. Neutrophil Isolation. Neutrophils were isolated from peripheral blood collected from volunteers with and without asthma by a modified standard protocol [29, 31, 32]. Briefly, 40 mL of blood was mixed with 10 mL acid citrate dextrose (ACD), 10 mL of phosphate buffered saline (PBS) (Gibco, Carlsbad, CA, USA), and 6 mL of 10% dextran (MP Biomedicals, Santa Ana, USA) and left for 20 minutes for sedimentation to occur at room temperature. The top layer was removed, overlaid on Ficoll Paque-PLUS (GE Healthcare, Little Chalfont, UK), and centrifuged at 490 g for 10 minutes.

Table 2: Smoking history and inhaled corticosteroid (ICS) use of healthy controls and asthmatics.

(a)

Healthy controls			
Age	Sex	Smoking status	Pack years
66	Male	Nonsmoker	0
66	Male	Nonsmoker	0
62	Female	Ex-smoker	15
70	Male	Nonsmoker	0
63	Female	Nonsmoker	0
67	Male	Nonsmoker	0
61	Female	Nonsmoker	0
67	Male	Nonsmoker	0
20	Male	Nonsmoker	0

(b)

Asthma, well-controlled*					
Age	Sex	Smoking status	Pack years	Daily ICS BDP-HFA equivalent (μg/day)	ICS in last 12 months in BDP-HFA equivalent (μg/day)
69	Female	Nonsmoker	0	0	200 (during exacerbations)
71	Male	Ex-smoker	18	200	—
75	Male	Nonsmoker	0	0	—
63	Male	Ex-smoker	0.5	1000	2000 (when needed)
52	Male	Nonsmoker	0	0	200† (exercise only)
61	Female	Nonsmoker	0	0	200 (in the last 12 months, ceased 3 months ago)
63	Male	Current smoker	43	0	200 (in high humidity, not used in the past months)
73	Male	Ex-smoker	21	1000	—
28	Male	Nonsmoker	0	125	—
69	Female	Nonsmoker	0	100†	200† (when sick)
65	Female	Nonsmoker	0	250	500 (when sick)

(c)

Asthma, suboptimally controlled*					
Age	Sex	Smoking status	Pack years	Daily ICS BDP-HFA equivalent (μg/day)	Oral steroid use in last 12 months
62	Male	Ex-smoker	0.2	800	Yes
71	Female	Ex-smoker	0.6	400	Yes
74	Male	Ex-smoker	4.5	1000	No
47	Male	Nonsmoker	0	1000	No
73	Female	Nonsmoker	0	1000	Yes
52	Male	Ex-smoker	7.15	200	No
59	Male	Nonsmoker	0	500	No

*See Section 2 for inclusion criteria.
†ICS-only inhaler; all other patients using ICS/LABA consistent with Australian prescribing trends [37].
BDP-HFA: beclometasone dipropionate (hydrofluoroalkane propellant).
ICS: inhaled corticosteroid.

The supernatant was discarded and the cell pellet of granulocytes was resuspended in sterile water for 30 seconds to lyse remaining red blood cells before osmolarity was reestablished with equal parts of 2x PBS. Cells were then incubated for 30 minutes at 4°C with CD16 magnetic beads (Miltenyi Biotec, Bergisch, Germany) before running through a magnetic column as per the manufacturer's instructions. Previous optimisation of the protocol showed typical purity was 99% or greater by a haematoxylin and eosin stain. The main contaminating cell was eosinophils (<1%).

2.4. RV16. RV16 was generously donated by Professor Sebastian Johnston, Imperial College, London, UK. RV16 was grown in HeLa cells by standard procedures and infectivity titre determined by a titration assay as described [33].

2.5. Stimulation of Neutrophils with Toll-Like Receptor (TLR) Agonists and RV16. Neutrophils were resuspended in 1% fetal bovine serum (FBS) (Glendarach Biologicals, Melbourne, Australia), 1% 1 M HEPES (Gibco), and 1% penicillin/streptomycin RPMI 1640 (Gibco) at 1×10^6 cells/mL. Cells were left unstimulated (negative control) or stimulated with EC_{50} concentrations of each TLR agonist based on dose-response curves generated for CXCL8 release (data not shown): 1 μg/mL LPS (Sigma Aldrich, St. Louis, MO, USA), 3 μg/mL imiquimod (Invivogen, San Diego, USA), 1.5 μg/mL R848 (Invivogen), and 10 μg/mL poly I:C (Sigma Aldrich), except fMLF (Sigma Aldrich) (100 nM) which was based on previous reports [34]. Neutrophils were also stimulated with RV16 at a multiplicity of infection (MOI) of 1 infectious particle per cell as previously published [25, 35]. Cells were incubated at 37°C with 5% CO_2 for 1 hour for NE and MMP-9 measurements or 24 hours for CXCL8 measurements. Cell-free supernatant and neutrophils cell pellets were collected and stored at −80°C for analysis.

2.6. CXCL8 Enzyme-Linked Immunosorbent Assay (ELISA). CXCL8 production was measured using a sandwich ELISA in duplicate. Specific ELISA kits from R&D Systems (Minneapolis, USA) were used according to the manufacturer's instructions. Detection limit was 15.6 pg/mL.

2.7. Neutrophil Elastase (NE) Activity Assay. NE activity was measured in duplicate using a fluorescence assay from Cayman Chemicals (Ann-Arbor, USA) according to the manufacturer's instructions. Fluorescence readings from samples were compared to a standard curve of known concentrations of NE to determine the concentration. Detection limit was 3.1 ng/mL.

2.8. MMP-9 Zymography. A bicinchoninic acid assay (Sigma Aldrich) was run for all samples according to the manufacturer's instructions to obtain the total protein concentration. Zymography was carried out according to previously published methods [36]. Briefly, 200 ng of total protein was loaded into each lane of a 1% gelatin polyacrylamide gel. The gel was run and then proteinases were activated in a $CaCl_2$ activation buffer overnight before staining with Coomassie brilliant blue dye. Bands were determined to be pro-MMP-9 using size markers and MMP-9 standards. Densitometry was performed with Carestream Molecular Imaging Software on images taken on a Kodak Image Station from Integrated Sciences (Chatswood, Australia) to determine the relative fold change compared to media control.

2.9. Data and Statistical Analysis. For statistical analysis, data was normalised ($\log_{10}$) before normality tests were conducted (Kolmogorov-Smirnov, D'Agostino and Pearson, and Shapiro-Wilk normality tests; GraphPad Prism 6). They were deemed to have a normal distribution if they passed one of the three normality tests. A paired t-test or one-way analysis of variance (ANOVA) with Dunnett's posttest was performed if the data followed a normal distribution or a Wilcoxon matched t-test or Friedman test with Dunn's multiple comparison test if data were nonparametric. Two-way ANOVA with Tukey's posttest was performed for comparisons between healthy controls, well-controlled asthmatic, and suboptimally controlled asthmatic. For some data sets, correlation analysis was performed. Significant changes were identified where $p < 0.05$.

3. Results

3.1. Patient Characteristics. The clinical characteristics for the study population are detailed in Tables 1 and 2. Patients were all age and gender matched with the mean age of approximately 60 years and each group consisted predominately of males. By definition, mean ACQ was different between the two asthma groups (good symptom control: mean 0.32, suboptimal symptom control: mean 1.33) There were no differences in FEV_1% predicted between the three patient groups; however FEV_1/FVC% ratio was significantly less in patients with well-controlled and suboptimally controlled asthma compared to healthy controls (Table 1). The majority of participants with well-controlled asthma were taking a short-acting β_2 agonist (82%) (Table 1) with only 55% taking an ICS containing inhaler daily (Table 2). However, all but 1 patient in this group took combination therapy intermittently in the past 12 months (Table 2). All participants with suboptimal asthma symptom control were taking a short-acting β_2 agonist (Table 1) along with combination therapy (Table 2).

3.2. Differential CXCL8 Release from Neutrophils from Asthmatics. All bacterial and viral mimetics including LPS, fMLF, imiquimod, R848, and poly I:C induced significant CXCL8 release from neutrophils isolated from healthy controls and well-controlled and suboptimally controlled asthmatics (Figures 1(a) and 1(b)). Interestingly, RV16 induced CXCL8 only in neutrophils from well-controlled and suboptimally controlled asthmatics (Figure 1(c)). Neutrophils from suboptimally controlled asthmatics had a deficient CXCL8 response to fMLF of approximately half when compared to healthy controls (Figure 1(a)). There was also a trend for neutrophils derived from well-controlled asthmatics to release less CXCL8 in response to fMLF which was of similar magnitude to neutrophils from suboptimally controlled asthmatics (Figure 1(a)). All other stimulants induced similar production of CXCL8 between all three groups.

3.3. Differential NE Release from Neutrophils from Asthmatics. fMLF was the only pathogen mimetic to induce NE from neutrophils in all three groups (Figure 2(a)). Interestingly, we found that, at baseline, neutrophils from well-controlled asthmatics had enhanced NE release compared to healthy controls (Figure 2(a)). Furthermore, this difference was also found when stimulated with fMLF (Figure 2(a)). RV16 did not induce NE release from neutrophils in any of the three groups.

3.4. Differential MMP-9 Release from Neutrophils from Asthmatics. LPS, fMLF, and imiquimod induced MMP-9 release from neutrophils in all three groups (Figures 3(a) and 3(b)).

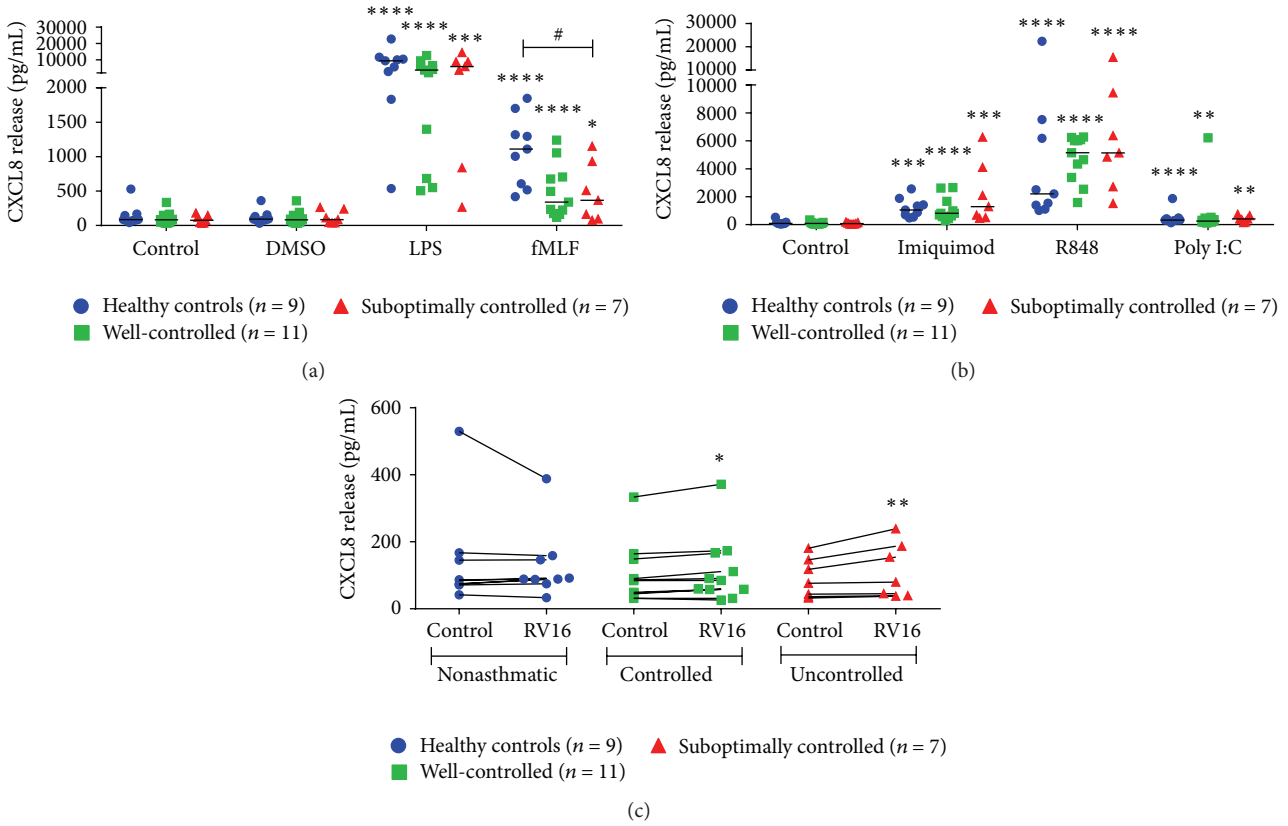

FIGURE 1: CXCL8 release from neutrophils stimulated with bacterial and viral mimetics and RV16. CXCL8 release from healthy controls (blue circles, $n = 9$), well-controlled asthmatic (green squares, $n = 11$), and suboptimally controlled asthmatic (red triangles, $n = 7$) neutrophils stimulated with (a) bacterial compounds: lipopolysaccharide (LPS), f-Met-Leu-Phe (fMLF), and DMSO (vehicle control), (b) viral surrogates: imiquimod, R848, and polyinosinic:polycytidylic acid (poly I:C), and (c) RV16 after 24 hours. Raw data is presented as a scatter plot with median. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared to unstimulated control. $^{\#}p < 0.05$ between indicated groups.

Similar to CXCL8 data, fMLF stimulated neutrophils from well-controlled and suboptimally controlled asthmatics had a deficient MMP-9 response compared to healthy controls (Figure 3(a)). Interestingly, R848 selectively induced MMP-9 in neutrophils derived from well-controlled and suboptimally controlled asthmatics, but not in healthy controls (Figure 3(b)). RV16 did not induce MMP-9 release from neutrophils in any of the three groups investigated.

3.5. FEV_1% Predicted and FEV_1/FVC% Ratio Correlated with fMLF Induced CXCL8. To further investigate if the differences in fMLF induced CXCL8 and MMP-9 in controlled and uncontrolled asthmatics were related to airway obstruction, we performed correlation analysis. We found that FEV_1% predicted and FEV_1/FVC% ratio positively correlated with fMLF induced CXCL8 release (Figures 4(a) and 4(b)) but not fMLF induced MMP-9 release (Figures 4(c) and 4(d)). There was no correlation between ACQ and fMLF induced CXCL8 ($r = -0.318$, 0.106) or MMP-9 ($r = -0.181$, $p = 0.367$). No correlations were found between pack year history and fMLF induced CXCL8 ($r = -0.04$, $p = 0.84$) or basal CXCL8 ($r = -0.07$, $p = 0.74$). Similarly, no correlations were found between daily ICS dose and fMLF induced CXCL8 ($r = -0.15$, $p = 0.45$) or basal CXCL8 ($r = 0.12$, $p = 0.55$). We also found no correlation between basal CXCL8 release and FEV_1% predicted ($r = 0.028$, $p = 0.889$) or FEV_1/FVC% ratio ($r = 0.029$, $p = 0.884$).

4. Discussion

In this study we found that neutrophils from patients with asthma respond differently to fMLF compared to healthy controls. However, we did not see differences between neutrophils from patients with well- versus suboptimally controlled asthma. RV16 induced CXCL8 and R848 induced MMP-9 occurred in only neutrophils from well- and suboptimally controlled asthmatic groups but not in neutrophils from healthy controls. fMLF stimulation resulted in a deficient MMP-9 production in neutrophils from well-controlled asthmatics and a deficient CXCL8 and MMP-9 in suboptimally controlled asthmatics. In addition, NE was differentially regulated and was constitutively elevated in well-controlled asthmatics. This increased constitutive release most likely accounts for the difference observed in fMLF stimulated neutrophils from well-controlled asthmatics. We also found that fMLF induced CXCL8 and MMP-9 release correlated with lung function but not ACQ, smoking history, or ICS use.

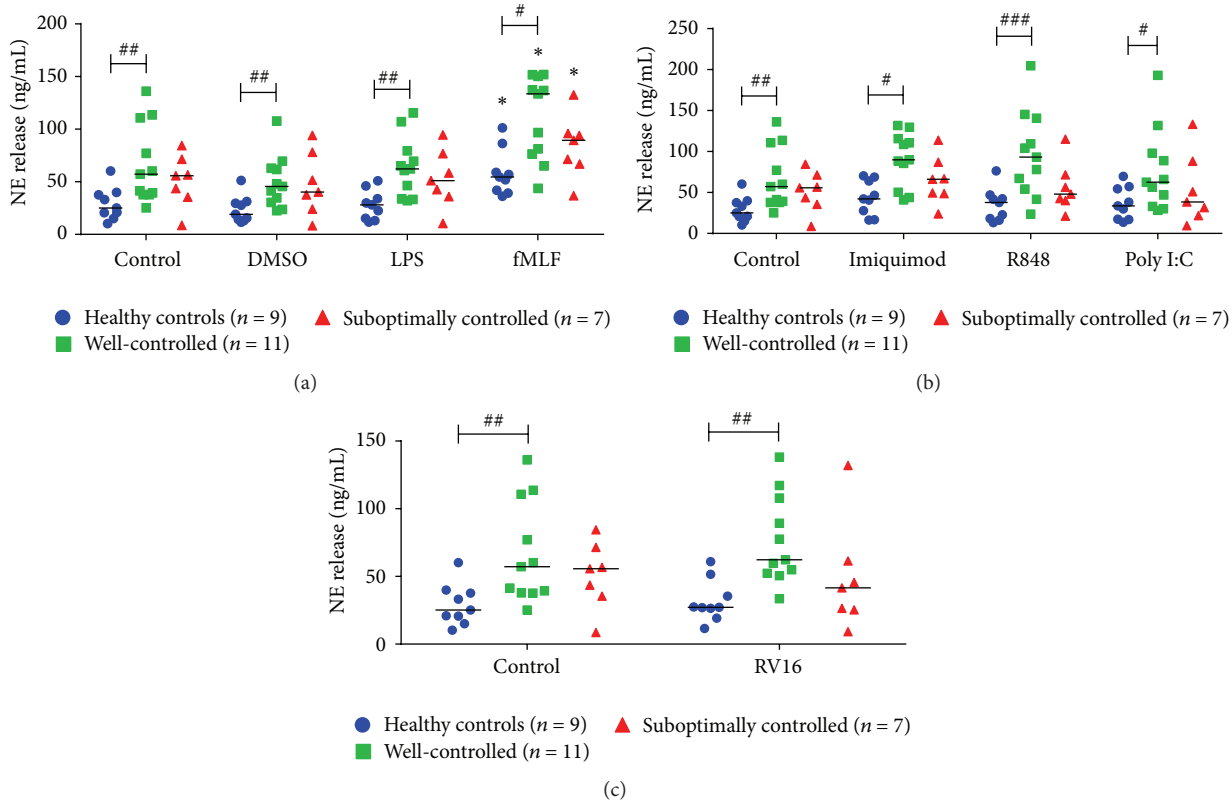

FIGURE 2: NE release from neutrophils stimulated with bacterial and viral mimetics and RV16. NE release from healthy controls (blue circles, $n = 9$), well-controlled asthmatic (green squares, $n = 11$), and suboptimally controlled asthmatic (red triangles, $n = 7$) neutrophils stimulated with (a) bacterial compounds: lipopolysaccharide (LPS), f-Met-Leu-Phe (fMLF), and DMSO (vehicle control), (b) viral surrogates: imiquimod, R848, and polyinosinic:polycytidylic acid (poly I:C), and (c) RV16 after 1 hour. Raw data is presented as a scatter plot with median. $^{*}p < 0.05$ compared to unstimulated control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ between indicated disease groups.

As airway epithelial cells and smooth muscle cells are fundamentally altered in asthma [21–24], even when grown for several cycles *in vitro* where they are deprived of altered signals in an asthmatic airway, we hypothesised that antimicrobial functions would similarly be dysfunctional in neutrophils from suboptimally controlled asthma. We hypothesised that there would be deficient immune responses in circulating neutrophils from people with suboptimally controlled asthma which potentially could lead to more severe or long lasting respiratory infections and ultimately an exacerbation. This study provides valuable and novel insights into circulating neutrophilic inflammatory responses.

In this study we were interested in investigating if neutrophils are already dysfunctional prior to entering the airway tissue in patients with asthma which could be intensified with altered inflammatory signals. As such, we chose to use peripheral blood neutrophils which we believe to be appropriate for investigation of possible differences in these cells. Since neutrophils are released into the circulation from the bone marrow we believe this cell population best reflects neutrophil function that has not had further differentiation signals provided during extravasation and in the airway lumen.

RV is a major precipitant of viral-induced exacerbations in asthma [8]. Neutrophils migrate into the airways during RV infections [38, 39] but their role in antiviral immunity remains unclear as is their ability to become infected with RV despite expressing ICAM-1 [40], the attachment protein for the serotype of RV used in this study. It is not clear why RV induced CXCL8 in this study. In the absence of replication we have previously shown that RV binding to ICAM-1 is sufficient to induce cytokine release in some [41] but not all lung cells [42]. Toll-like receptors (TLRs), particularly TLR 3, TLR 7, and TLR 8, detect viruses and usually their activation leads to typical innate activation. Potentially if RV is phagocytosed, TLR 3, TLR 7, and TLR 8 present on phagocytic vesicles may be activated. Alternatively cell surface TLR 3 [43, 44] may detect the presence of the virus.

Our finding that RV16 can induce CXCL8 in asthmatic neutrophils is novel, although the clinical relevance for the small induction observed here is questionable and needs to be interpreted with caution. However, the titre of RV *in vivo* is likely to be much higher than we used *in vitro* (reported 1000 TCID/mL in nasal lavage fluid [45]); therefore we speculate that a greater response to RV may occur *in vivo*. It is also plausible that patients with more severe disease are more sensitive to relatively small changes in inflammatory

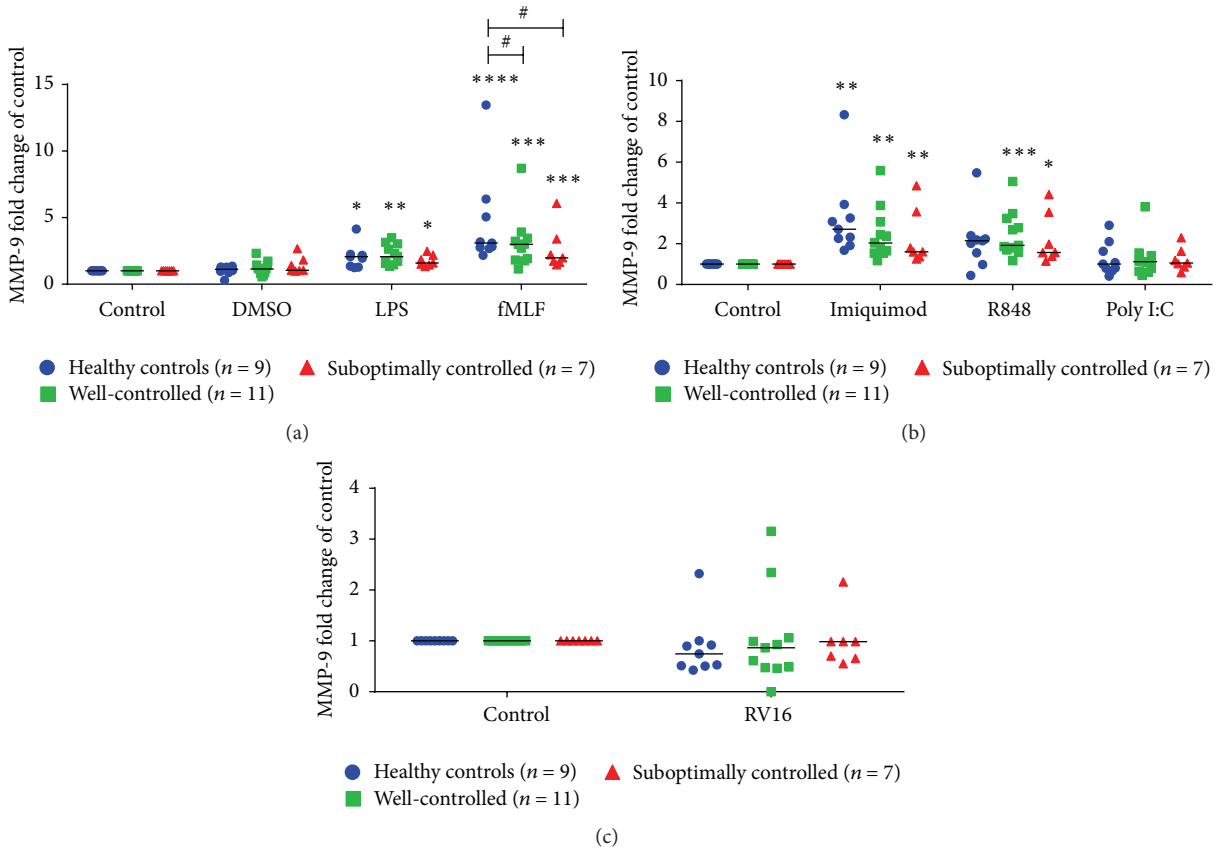

FIGURE 3: MMP-9 release from neutrophils stimulated with bacterial and viral mimetics and RV16. MMP-9 release from healthy controls (blue circles, $n = 9$), well-controlled asthmatic (green squares, $n = 11$), and suboptimally controlled asthmatic (red triangles, $n = 7$) neutrophils stimulated with (a) bacterial compounds: lipopolysaccharide (LPS), f-Met-Leu-Phe (fMLF), and DMSO (vehicle control), (b) viral surrogates: imiquimod, R848, and polyinosinic:polycytidylic acid (poly I:C), and (c) RV16 after 1 hour. Raw data is presented as a scatter plot with median. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared to unstimulated control. $^{\#}p < 0.05$ between indicated disease groups.

cytokine production due to the cumulative effect of a greater number of neutrophils present. Dysregulation in CXCL8 induction by RV16 could play a role in the pathogenesis of asthma exacerbations and persistent airway neutrophilia but this requires further investigation.

fMLF, a bacterial derived protein and ligand for the fMLF receptor, stimulates neutrophils to migrate, produce inflammatory mediators, and release granules and reactive oxygen species [46]. We observed stimulant and disease specific changes in the response to fMLF. We believe these changes are not related to fMLF receptor expression since in the same patients with well-controlled asthma; fMLF induced CXCL8 release was approximately half the response of healthy controls but NE release was augmented.

NE and MMP-9 are both proteases and are found in neutrophil azurophil and gelatinase granules, respectively [47]. Like all proteases, tight regulation is required to ensure localisation of the enzymes to the area of infection; otherwise tissue damage can occur. We observed that, even under the same stimulation conditions, NE and MMP-9 release were differentially regulated, that is, a deficient fMLF induced MMP-9 response but an augmented fMLF induced NE response in neutrophils from well-controlled asthmatics. This differential regulation could be due to the location of these products in different types of granules and their differing propensity to be released from neutrophils under certain stimulation conditions. However, further investigation of these mechanisms was outside the scope of this study and could be the subject for future studies.

Interestingly, we found similar neutrophil responses between patients with suboptimally controlled asthma and well-controlled asthma, particularly with deficient fMLF induced CXCL8 and MMP-9 release. Neutrophil function is known to decline with age and these changes include decreased phagocytic ability [48, 49], reduction in degranulation [50], and reduced capacity to generate reactive oxygen species [48]. In previous work we found that neutrophils from asthmatics with a mean age of 35 years had a greater propensity to release CXCL8 with R848 stimulation compared to nonasthmatic controls; however, other stimulants which included fMLF were similar between the two groups [25]. In this study where participants had a mean age of 62 years, we did not observe enhanced R848 induced CXCL8 from neutrophils, rather similar levels between both asthmatic groups and healthy controls. In addition, we noted deficient CXCL8 and MMP-9 release with fMLF stimulation which

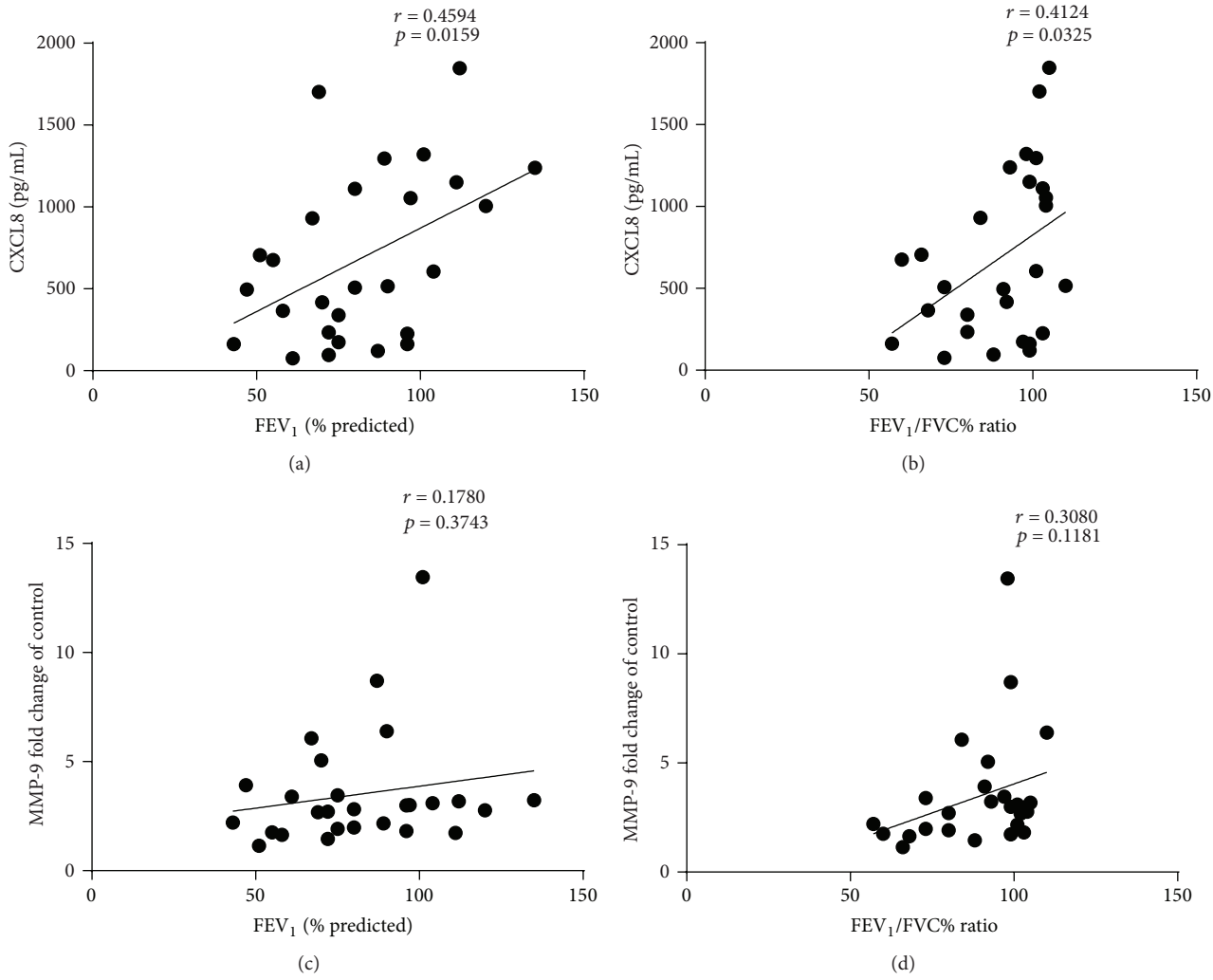

Figure 4: Correlation of FEV_1 and FEV_1/FVC with fMLF induced CXCL8 and MMP-9 from neutrophils. Correlations of fMLF induced (a-b) CXCL8 release and (c-d) MMP-9 with FEV_1 (a-c) and FEV_1/FVC ratio (b-d).

may suggest that decline in neutrophil function is greater in those with disease, particularly in recognition of bacteria.

Studies have reported CXCL8 levels in the airways inversely correlate with FEV_1 in asthmatic individuals [3, 51, 52]. These studies measured CXCL8 in the bronchial alveolar lavage (BAL) and induced sputum which indicate the total inflammatory mediator load in the airways but give little indication of the source. In this study we found that basal CXCL8 release from neutrophils does not correlate with lung function, suggesting that neutrophils may not be the main source of this cytokine in BAL. However, fMLF induced CXCL8 positively correlated with both FEV_1 and FEV_1/FVC. fMLF stimulates neutrophils via the FPR1 receptor. Interestingly annexin A1 also activates the fMLF receptor [53]. As corticosteroid induced annexin A1 is a major anti-inflammatory mechanism it is interesting to speculate that the responsiveness to fMLF may also indicate steroid insensitivity.

Our study has several limitations; the number of participants is small and the patients with suboptimal asthma control may have been quite heterogenous. Few participants had an ACQ > 1.5, which is the cut point used to be confident that asthma control is poor [30]. As we do not have eosinophil counts there is potential that the suboptimally controlled asthma group could contain steroid refractory eosinophilic asthmatics. These patients are distinct from neutrophilic refractory asthmatics as they have eosinophilia despite high dose steroid therapy and respond to anti-IL-5 antibody therapy [54, 55]. Detailed phenotyping and endotyping of asthma are areas of interest to help personalise treatment options [56]. Our study is limited in this respect and future studies with larger population groups could address this question. It is also becoming clear that neutrophils are a nonhomogenous population of cells that carry out various functions ranging from the classical proinflammatory response to an immune modulatory response [57]. Investigation in this area was out of the scope of our study but would be of interest to closely investigate neutrophil subtypes in different asthmatic populations.

In conclusion, no significant differences were seen in neutrophil function between patients with well- and suboptimally controlled asthma and therefore it is unlikely that neutrophil dysregulation drives asthma control. However, neutrophils from people with asthma appear to have different responses to pathogenic stimuli compared to healthy controls. This dysfunction may contribute to persistent or greater susceptibility to infection in asthmatics and is likely to be associated with airway obstruction.

Abbreviations

ACQ: Asthma control questionnaire
BDP-HFA: Beclometasone dipropionate (hydrofluoroalkane propellant)
CXCL8: Interleukin 8
FEV_1: Forced expiratory volume in 1 second
fMLF: f-Met-Leu-Phe
FVC: Forced vital capacity
ICS: Inhaled corticosteroid
LPS: Lipopolysaccharide
MMP-9: Matrix metalloproteinase-9
NE: Neutrophil elastase
RV: Rhinovirus.

Ethical Approval

This study was approved by Human Research Ethics Committee, The University of Sydney.

Consent

Written informed consent was obtained from all volunteers.

Conflict of Interests

The authors declared no conflict of interests regarding this paper.

Authors' Contribution

Francesca S. M. Tang, Peter G. Gibson, Gloria J. Foxley, Janette K. Burgess, Katherine J. Baines, and Brian G. Oliver provided conception and design of the project. Francesca S. M. Tang and Gloria J. Foxley carried out recruitment. Francesca S. M. Tang completed all cell biology, laboratory work, and data analysis. All authors contributed to the preparation of the paper.

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Research Article

Caffeine Mitigates Lung Inflammation Induced by Ischemia-Reperfusion of Lower Limbs in Rats

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Reperfusion of ischemic limbs can induce inflammation and subsequently cause acute lung injury. Caffeine, a widely used psychostimulant, possesses potent anti-inflammatory capacity. We elucidated whether caffeine can mitigate lung inflammation caused by ischemia-reperfusion (IR) of the lower limbs. Adult male Sprague-Dawley rats were randomly allocated to receive IR, IR plus caffeine (IR + Caf group), sham-operation (Sham), or sham plus caffeine ($n = 12$ in each group). To induce IR, lower limbs were bilaterally tied by rubber bands high around each thigh for 3 hours followed by reperfusion for 3 hours. Caffeine (50 mg/kg, intraperitoneal injection) was administered immediately after reperfusion. Our histological assay data revealed characteristics of severe lung inflammation in the IR group and mild to moderate characteristic of lung inflammation in the IR + Caf group. Total cells number and protein concentration in bronchoalveolar lavage fluid of the IR group were significantly higher than those of the IR + Caf group ($P < 0.001$ and $P = 0.008$, resp.). Similarly, pulmonary concentrations of inflammatory mediators (tumor necrosis factor-α, interleukin-1β, and macrophage inflammatory protein-2) and pulmonary myeloperoxidase activity of the IR group were significantly higher than those of the IR + Caf group (all $P < 0.05$). These data clearly demonstrate that caffeine could mitigate lung inflammation induced by ischemia-reperfusion of the lower limbs.

1. Introduction

Lower limb ischemia can be caused by a variety of clinical conditions, including critical limb ischemia, abdominal aortic aneurysm, and traumatic arterial injury [1–3]. Therapies that can restore perfusion to the ischemic limb(s) are performed to reduce injury caused by ischemia. However, reperfusion of the ischemic limb(s) can in turn induce inflammation and cause remote organ injury [4–6]. In this regard, the lung is one of the organs most vulnerable to remote injury subsequent to ischemia-reperfusion [2, 4, 6].

Caffeine (1,3,7-trimethylxanthine) is a widely used psychostimulant. Caffeine alone is used clinically in the treatment of headache, respiratory depression in neonates, obesity, and postprandial hypotension [7]. These above-mentioned effects of caffeine are mediated by inhibition of methylxanthine-sensitive adenosine receptors [7]. In addition, caffeine has been shown to possess potent anti-inflammatory capacity [8, 9]. Using animal models, previous studies confirmed that caffeine (especially high dose caffeine) exerted significant therapeutic effects against traumatic brain injury [10] and oleic oil-induced lung injury [11]. Caffeine was also shown to exert protective effects against myocardial ischemia-reperfusion [12].

To date, the question of whether caffeine could be protective of lung tissues against the adverse effects of ischemia-reperfusion of the lower limbs remains unstudied. To elucidate further, we thus conducted this study. This

systematic study used an established anesthetized rodent model of ischemia-reperfusion of the lower limbs [4, 13] to determine if systemic application of caffeine at reperfusion would mitigate anatomical and biochemical markers of lung inflammation and pathology.

2. Materials and Methods

This animal study was approved by the Institutional Animal Use and Care Committee of Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation (102-IACUC-014). Rats were treated according to National Institutes of Health guidelines. A total of 48 adult male Sprague-Dawley rats (200 g to 250 g; BioLASCO Taiwan Co., Ltd., Taipei, Taiwan) were used in this study. All rats were fed a standard laboratory chow and water at liberty until the experimental day.

2.1. Animal Preparation. All rats were anesthetized with an intraperitoneal (ip) injection of a mixture of zoletil (40 mg/kg; Virbac, Carros, France) and xylazine (Rompun TS, Bayer, Leverkusen, Germany) and were placed in a supine position. The right carotid artery was cannulated with a polyethylene (PE-50) catheter for continuous hemodynamic monitoring and blood drawing. A tracheostomy was performed and a 16-gauge angiocatheter was inserted as a tracheostomy tube. Blood pressure and respiratory rate were continuously monitored throughout the experiments. Supplemental doses of zoletil/xylazine mixture (13/3 mg/kg, ip) were given every 30–60 minutes until the end of the study to ensure and maintain adequate anesthesia.

2.2. Experimental Protocols. The protocol of lower limb ischemia-reperfusion injury was modified from previously published reports [4, 13]. In brief, bilateral lower limb ischemia-reperfusion was performed by applying rubber band tourniquets high around each thigh for 3 hours followed by reperfusion for 3 hours. Half of the rats received the lower limb ischemia-reperfusion injury protocol. To control for the effects of manipulations, the remaining rats received a sham-operation, that is, anesthesia, carotid artery cannulation, and tracheostomy, but no introduction of the rubber band tourniquets and limb ischemia-reperfusion.

2.3. Experimental Groups. All rats were randomly assigned to one of the four experimental groups ($n = 12$ in each group): the sham-operation (Sham), the sham plus caffeine (Sham + Caf), the lower limbs ischemia-reperfusion (IR), and the IR plus caffeine (IR + Caf) groups. Rats of the Sham + Caf and the IR + Caf groups received caffeine (50 mg/kg, ip; Sigma-Aldrich, St. Louis, MO, USA) immediately after reperfusion. The dose of caffeine (50 mg/kg ip) was chosen according to a previous study demonstrating that caffeine at this dose could mitigate oleic acid-induced lung injury in mice [11]. To control for the effects of treatment vehicle, rats of the Sham and the IR group received normal saline (1.0 mL, ip) at comparable time point. After 3 hours of reperfusion, all rats were euthanized with neck dislocation.

2.4. Lung Tissue Collection and Bronchoalveolar Lavage. Thoracotomy was performed to facilitate lung tissue harvesting. The left main bronchus was tied and the left lungs were removed. The left lung tissues were snap-frozen in liquid nitrogen and stored at −80°C for subsequent analysis. To facilitate histological analysis, the right lung tissues of six rats from each group were infused with 4% formaldehyde through the tracheostomy tube and then removed. To facilitate bronchoalveolar lavage fluid (BALF) analysis, the right lungs of the other six rats from each group were lavaged five times with 3 mL sterile normal saline, as we have previously reported [13, 14]. The BALF was then collected. To maximize the efficacy of BALF collection, suction was performed twice after each lavage. The five fractions of BALF from each rat were pooled together and saved for the subsequent analysis.

2.5. Histological Analysis. The formaldehyde-infused lung tissues were embedded in paraffin wax, serially sectioned, and then stained with hematoxylin and eosin. Lung tissue inflammation was evaluated using a light microscope by a pathologist who was blind to this study. Histologic characteristics, including edematous change of the alveolar wall, hemorrhage, vascular congestion, and polymorphonuclear leukocytes (PMN) infiltration, were used to evaluate lung inflammation, according to our previous report [14].

2.6. Total Cells Number and Protein Concentration in BALF. An aliquot of the pooled BALF (50 μL) from each rat was diluted 1 : 1 with trypan blue dye (Life Technologies, Grand Island, NY, USA) and the total cells number was counted using a standard hemocytometer, using our previously reported protocol [13, 14]. The remaining pooled BALF from each rat was centrifuged (3000 rpm for 5 minutes at 15°C) and then the supernatants were collected. The protein concentration of the BALF supernatant was analyzed using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA), as directed by the manufacturer's protocol. The BALF samples were analyzed in triplicate.

2.7. Inflammatory Mediators and Myeloperoxidase (MPO) Activity. The frozen lung tissues were processed according to our previous reports [13, 14]. For inflammatory mediators, frozen lung tissues were weighed and homogenized with a tissue homogenizer (MICCRA D-1, ART Prozess & Labortechnik GmbH & Co. KG, Müllheim, Germany) in 5 volumes of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris-HCl, pH 7.5; all chemicals were from Sigma-Aldrich) and incubated at 4°C in RIPA buffer. Following centrifugation (14,000 rpm at 4°C for 20 minutes), the tissue supernatants were collected. After measuring the protein concentration using a BCA protein assay kit (Pierce), the concentrations of cytokines (e.g., tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)) and chemokine (e.g., macrophage inflammatory protein-2, MIP-2) in the tissue supernatants were analyzed in triplicate using commercial enzyme-linked immunosorbent assay (ELISA) kits (ELISA kits for TNF-α and IL-1β, Pierce; MIP-2 ELISA kit; R&D Systems, Inc.,

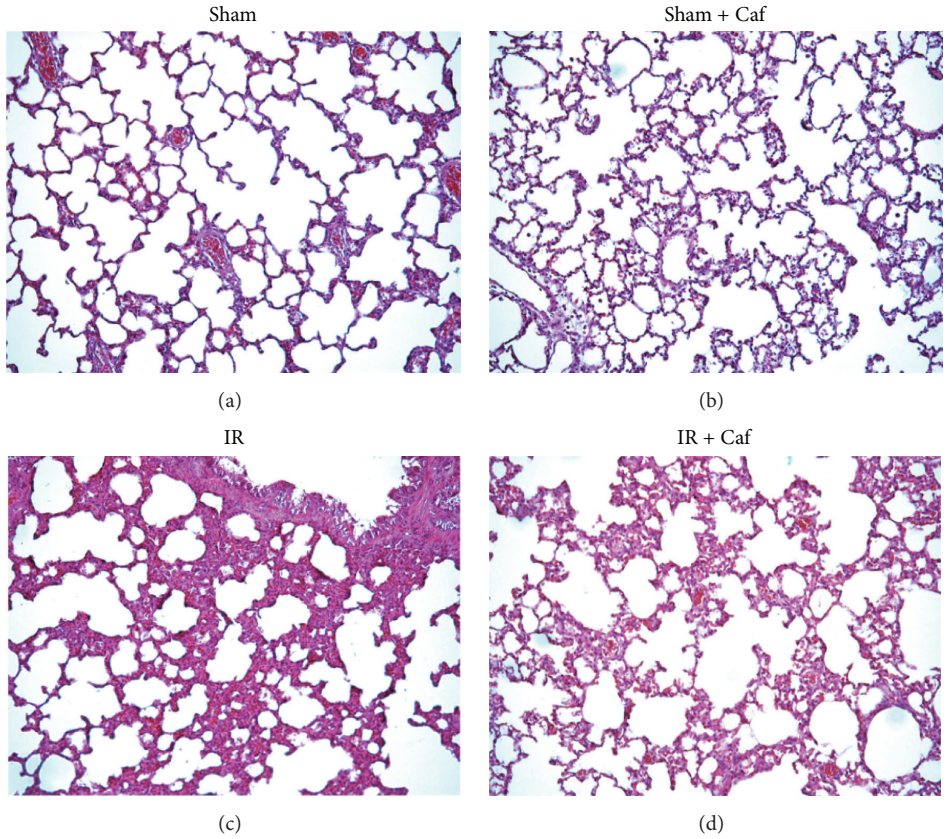

Figure 1: Representative microscopic findings of the lung tissues stained with hemotoxylin & eosin (100x). (a) The sham-operation (Sham) group revealed normal to mild lung inflammation characteristics. (b) The sham plus caffeine (Sham + Caf) group revealed normal to mild lung inflammation characteristics. (c) The lower limb ischemia-reperfusion (IR) group revealed severe lung inflammation characteristics. (d) The IR plus caffeine (IR + Caf) group revealed mild to moderate lung inflammation characteristics. Rats of the Sham + Caf and the IR + Caf groups received caffeine (50 mg/kg, intraperitoneal injection) immediately after reperfusion. To control the effects of vehicle, rats of the Sham and the IR group received normal saline (1.0 mL, intraperitoneal injection) at comparable time point.

Minneapolis, MN, USA). ELISA was performed as per the manufacturers' protocols.

Pulmonary MPO activity from snap-frozen tissue was quantified, as per our previous reports [13, 14], to measure the activity of the infiltrated PMN, an indicator of lung inflammation [13]. Lung tissue samples were weighed and homogenized for 1 minute in 15 volumes of PE buffer (0.01 M KH_2PO_4 with 1 mM EDTA). Following homogenization and centrifugation (14,000 rpm at 4°C for 20 minutes), the pellets were collected and resuspended in 15 volumes of cetyltrimethylammonium bromide buffer (13.7 mM) with acetic acid (50 mM). The resuspended pellets were then sonicated and centrifuged (10,000 rpm for 15 minutes at 15°C). The supernatants were collected and incubated in a water bath for 2 hours at 60°C. MPO activity was measured using a MPO fluorometric detection kit (Enzo Life Science, Plymouth Meeting, PA, USA), according to the manufacturer's instructions. The samples were analyzed in triplicate. All chemicals were from Sigma-Aldrich.

2.8. Malondialdehyde (MDA) Assay. Snap-frozen lung tissue homogenates were assayed for MDA using thiobarbituric acid test, as per our previous published protocols [13, 14], to quantify the status of lipid peroxidation [15]. In brief, snap-frozen lung tissues were weighed and homogenized in 5 volumes of RIPA buffer on ice. After centrifugation (2000 rpm at 4°C for 10 minutes), the supernatants were collected and stored on ice. The MDA concentrations of the supernatants were measured using a commercial MDA assay kit (TBARS assay kit, Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer's instruction. The samples were also analyzed in triplicate.

2.9. Statistical Analysis. One-way analysis of variance with the Bonferroni-Dunn test was used for multiple comparisons. Data were presented as mean ± standard deviation. The significance level was set at 0.05. A commercial software package (SigmaStat for Windows, SPSS Science, Chicago, IL) was used for data analysis.

3. Results

3.1. Lung Histology Data. Histological analysis revealed normal to mild lung inflammation characteristics in the Sham and the Sham + Caf groups (Figures 1(a) and 1(b)). The

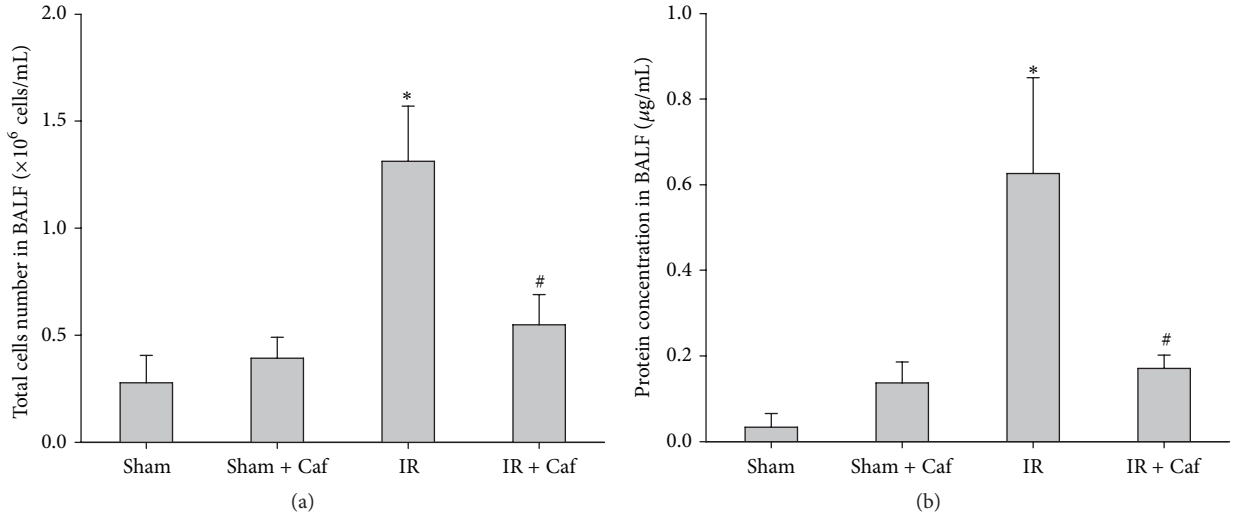

Figure 2: Total cells number (a) and protein concentration (b) in bronchoalveolar lavage fluid (BALF). Sham: the sham-operation group. Sham + Caf: the sham plus caffeine group. IR: the lower limb ischemia-reperfusion group. IR + Caf: the IR plus caffeine group. Rats of the Sham + Caf and the IR + Caf groups received caffeine (50 mg/kg, intraperitoneal injection) immediately after reperfusion. To control for the effects of the treatment vehicle, rats of the Sham and the IR group received normal saline (1.0 mL, intraperitoneal injection) at the comparable time point. One-way analysis of variance with the Bonferroni-Dunn test was used for multiple comparisons. The significance level was set at 0.05. Data were derived from 6 rats from each group and presented as mean ± standard deviation. $^*P < 0.05$: the IR group versus the Sham group. $^\#P < 0.05$: the IR + Caf group versus the IR group.

lung tissues of the IR group revealed severe inflammation characteristics (Figure 1(c)). Moreover, the lung tissues of the IR + Caf group revealed mild to moderate lung inflammation characteristics (Figure 1(d)).

3.2. BALF Data. In BALF from the Sham group, the total cells number was $0.28 \pm 0.13 \times 10^6$ cells/mL and the protein concentration was 0.03 ± 0.03 μg/mL (Figures 2(a) and 2(b)). The total cells number ($0.39 \pm 0.09 \times 10^6$ cells/mL) and protein concentration (0.14 ± 0.05 μg/mL) in BALF of the Sham + Caf groups were similar to those of the Sham group (Figures 2(a) and 2(b)). The total cells number ($1.31 \pm 0.26 \times 10^6$ cells/mL) and protein concentration (0.63 ± 0.22 μg/mL) of the IR group were significantly higher than those of the Sham group ($P < 0.001$ and =0.002, resp.; Figures 2(a) and 2(b)). Moreover, the total cells number ($0.55 \pm 0.14 \times 10^6$ cells/mL) and protein concentration (0.17 ± 0.03 μg/mL) of the IR + Caf group were significantly lower than those of the IR group ($P < 0.001$ and $P = 0.008$, resp.; Figures 2(a) and 2(b)).

3.3. Pulmonary Inflammatory Mediators and MPO Activity Data. In the Sham group, the pulmonary concentration of TNF-α was 25.8 ± 9.4 pg/mL, IL-1β was 121.8 ± 48.7 pg/mL, MIP-2 was 31.7 ± 17.2 pg/mL, and MPO activity was 880.7 ± 26.2 mU/mL (Figures 3(a)–3(d)). The pulmonary concentrations of TNF-α (14.0 ± 3.2 pg/mL), IL-1β (106.2 ± 53.5 pg/mL), and MIP-2 (38.9 ± 20.0 pg/mL) as well as the pulmonary MPO activity (882.8 ± 33.4 mU/mL) of the Sham + Caf group were similar to those of the Sham group (Figures 3(a)–3(d)). The pulmonary concentrations of TNF-α (163.5 ± 90.4 pg/mL), IL-1β (478.4 ± 213.3 pg/mL), and MIP-2 (909.3 ± 422.5 pg/mL) as well as the pulmonary MPO activity (1010.7 ± 38.8 mU/mL) of the IR group were significantly higher than those of the Sham group (all $P < 0.001$; Figures 3(a)–3(d)). In contrast, the pulmonary concentrations of TNF-α (36.4 ± 12.1 pg/mL), IL-1β (106.5 ± 38.4 pg/mL), and MIP-2 (83.2 ± 62.2 pg/mL) as well as the pulmonary MPO activity (864.7 ± 25.4 mU/mL) of the IR + Caf group were significantly lower than those of the IR group (all $P < 0.001$; Figures 3(a)–3(d)).

3.4. Pulmonary MDA Data. The pulmonary MDA concentration of the Sham group was 16.2 ± 0.8 units/gm tissue and the Sham + Caf group was 15.2 ± 0.6 units/gm tissue (Figure 4). The pulmonary MDA concentration (20.8 ± 1.1 units/gm tissue) of the IR group was significantly higher than that of the Sham group ($P < 0.001$; Figure 4). In contrast, the pulmonary MDA concentration (17.0 ± 0.8 units/gm tissue) of the IR + Caf group was significantly lower than that of the IR group ($P < 0.001$; Figure 4).

4. Discussion

The results of this study are consistent with previous studies [2, 4, 6] and confirmed that ischemia-reperfusion of the lower limbs can induce significant inflammation of the lung. Furthermore, the present study clearly demonstrates that in this rodent model caffeine mitigates lung inflammation induced by lower limbs ischemia-reperfusion although the underlying mechanisms were not investigated.

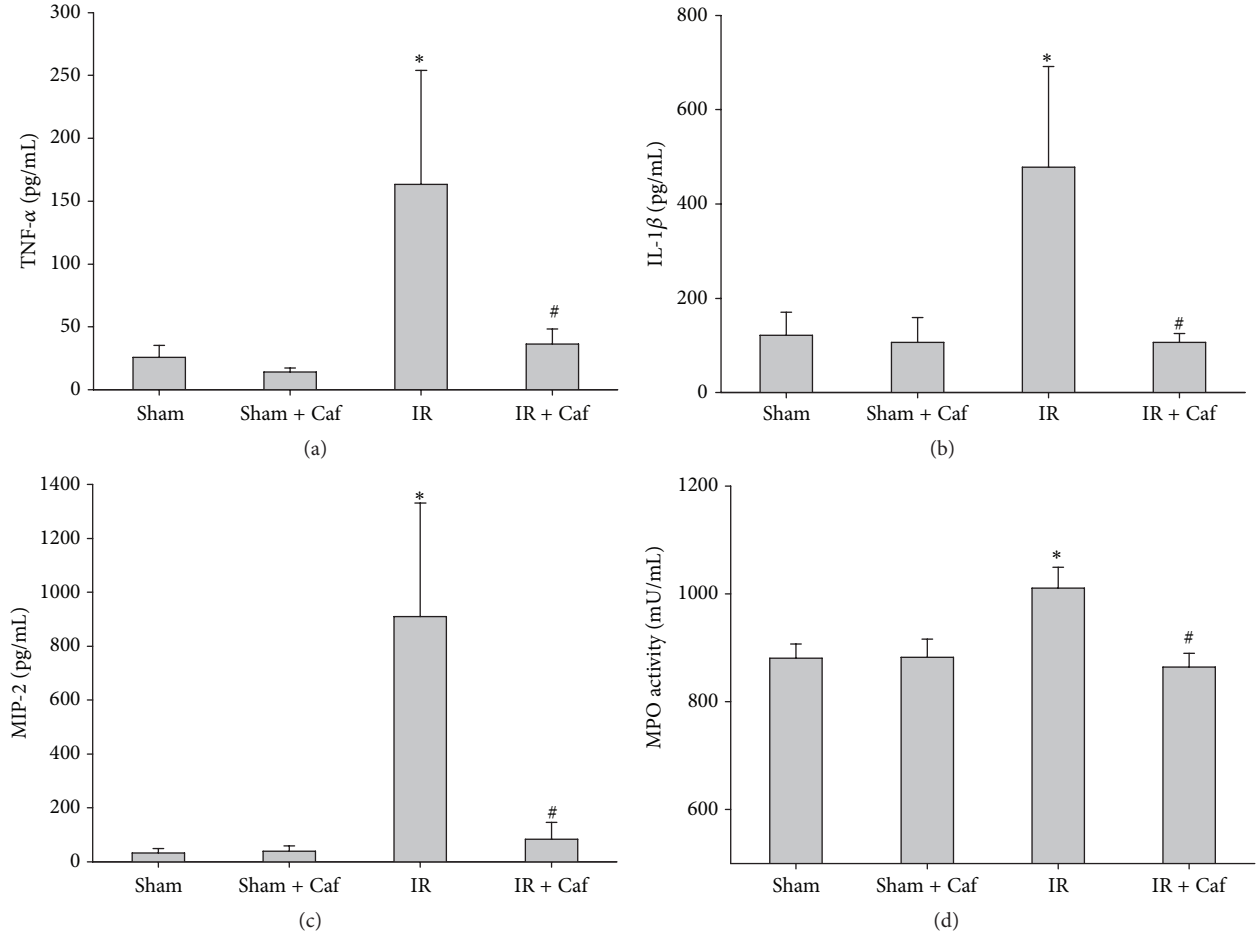

Figure 3: The pulmonary concentrations of (a) tumor necrosis factor-α (TNF-α), (b) interleukin-1β (IL-1β), (c) macrophage inflammatory protein-2 (MIP-2), and (d) myeloperoxidase (MPO) activity. Sham: the sham-operation group. Sham + Caf: the sham plus caffeine group. IR: the lower limb ischemia-reperfusion group. IR + Caf: the IR plus caffeine group. Rats of the Sham + Caf and the IR + Caf groups received caffeine (50 mg/kg, intraperitoneal injection) immediately after reperfusion. To control for the effects of treatment vehicle, rats of the Sham and the IR group received normal saline (1.0 mL, intraperitoneal injection) at the comparable time point. One-way analysis of variance with the Bonferroni-Dunn test was used for multiple comparisons. The significance level was set at 0.05. Data were derived from 6 rats from each group and presented as mean ± standard deviation. $^{*}P < 0.05$: the IR group versus the Sham group. $^{\#}P < 0.05$: the IR + Caf group versus the IR group.

It is well established that inflammation is a crucial mechanism in mediating remote organ injury induced by ischemia-reperfusion of the lower limbs [4, 13] but a mechanist appraisal of the protective effect of caffeine can, at this stage, only be alluded to indirectly from an extensive literature of related studies. Expression of inflammatory mediators is tightly regulated by the crucial upstream transcriptional factor nuclear factor-κB (NF-κB) [16]. Pervious data also indicated that ischemia-reperfusion can activate NF-κB expression [17]. As such, it is plausible that the protective, anti-inflammatory effects of caffeine in the lung may act, in part, through inhibition of NF-κB activation. This concept is supported by previous data that caffeine could inhibit NF-κB activation in endotoxin-stimulated microglia [18]. Interestingly, poly(ADP-ribose) polymerase- (PARP-) 1 is a cofactor for NF-κB mediated upregulation of inflammatory mediators [19]. However, in cultured epithelial and endothelial cells, caffeine metabolites, at physiological levels, inhibit PARP-1 [20]. In addition, cyclic AMP (cAMP) is a potent inhibitor of NF-κB [21]. Degradation of cAMP is tightly regulated by phosphodiesterases and inhibition of phosphodiesterases can increase the level of cAMP which in turn inhibits NF-κB activity [22]. Of relevance is the fact that caffeine is a nonselective phosphodiesterase inhibitor [23]. In light of the cited literature, we speculate that the mechanisms underlying the anti-inflammatory role of caffeine in ischemia-reperfusion of the lower limbs may also involve its effects on inhibiting PARP-1 and/or phosphodiesterases.

In addition to inflammation, oxidative stress also plays a crucial role in mediating the development of lung injury induced by ischemia-reperfusion of the lower limb [4, 13, 24, 25]. Previous studies demonstrated that ischemia-reperfusion of the lower limb significantly increased xanthine oxidase activity and promoted oxidant generation as well

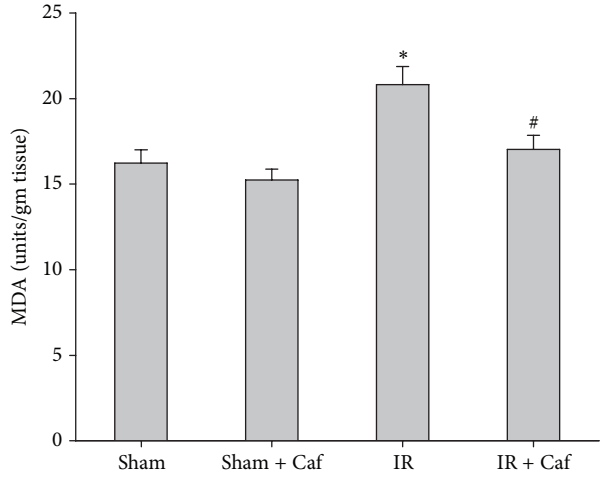

Figure 4: The pulmonary malondialdehyde (MDA) concentrations. Sham: the sham-operation group. Sham + Caf: the sham plus caffeine group. IR: the lower limb ischemia-reperfusion group. IR + Caf: the IR plus caffeine group. Rats of the Sham + Caf and the IR + Caf groups received caffeine (50 mg/kg, intraperitoneal injection) immediately after reperfusion. To control for the effects of treatment vehicle, rats of the Sham and the IR group received normal saline (1.0 mL, intra-peritoneal injection) at the comparable time point. One-way analysis of variance with the Bonferroni-Dunn test was used for multiple comparisons. The significance level was set at 0.05. Data were derived from 6 rats from each group and presented as mean ± standard deviation. $^{*}P < 0.05$: the IR group versus the Sham group. $^{\#}P < 0.05$: the IR + Caf group versus the IR group.

as lipid peroxidation while the application of antioxidants mitigated acute lung injury in this model [13, 24, 25]. A considerable body of evidence documents the direct free radical scavenging capacity of caffeine [26] while it has also been recognized that caffeine has the ability to enhance the expression of the upstream transcription factor nuclear factor-E2-related factor 2 (Nrf2) and the downstream antioxidant enzyme system, including superoxide dismutase (SOD) and catalase [27]. In our study, levels of pulmonary MDA, an indicator of lipid peroxidation, were significantly increased by ischemia-reperfusion of the lower limb and this increase was significantly attenuated by the application of caffeine, a result consistent with an antioxidant action of caffeine. As such, our results suggest that a component of the protective effects of caffeine may be derived from its actions on the pathways mediating oxidative stress.

In this rodent study, the protective effects of caffeine are clear. However, the question of whether the therapeutic effects of caffeine are dose-dependent remains unstudied. As previously mentioned, the dose of caffeine employed in this study was based upon the protective effect of 50 mg/kg caffeine (ip) on acute lung injury induced by oleic acid in mice [11]. Somewhat paradoxically in the same study, two lower doses of caffeine, 5 and 15 mg/kg, aggravated the lung injury induced by oleic acid [11]. Moreover, the therapeutic potential of low doses of caffeine, that is, 5 or 15 mg/kg, had also been investigated using a rodent model of traumatic brain injury [10], but these doses failed to modulate indices of traumatic brain injury [10]. Although the present study employed only a single high dose of caffeine, these inconsistent literature findings with respect to dose and ischemic model prompted us to perform a series of preliminary studies to test the effects of lower doses of caffeine (i.e., 10 or 25 mg/kg) on the modulation of the upregulation of pulmonary TNF-α induced by lower limb ischemia-reperfusion. Our preliminary data revealed that low doses of caffeine (10 or 25 mg/kg) exerted no significant modulation of the upregulation of pulmonary TNF-α in lower limbs ischemia-reperfusion (data not shown). Though more studies are needed before further conclusions can be made, nevertheless, these data clearly indicate that the significant anti-inflammatory effects of caffeine can only be observed with high dose.

To elucidate further, we are currently conducting a follow-up study to compare the therapeutic potentials between 100 mg/kg caffeine and 50 mg/kg caffeine using the same model. The preliminary data obtained from the follow-up study revealed that 100 mg/kg caffeine could significantly inhibit the increases in total cells number and protein concentration in BLAF induced by lower limb ischemia-reperfusion in rats (please see Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/361638). However, our preliminary data also revealed that total cells number and protein concentration in BLAF in rats receiving lower limb ischemia-reperfusion plus 100 mg/kg caffeine were not significantly different from those in rats receiving lower limb ischemia-reperfusion plus 50 mg/kg caffeine (Supplemental Figure 1). These data seem to suggest that the therapeutic potentials of 100 mg/kg caffeine and 50 mg/kg caffeine were similar. In line with this notion, we thus speculate that there may be a ceiling effect regarding the therapeutic potential of high dose caffeine in mitigating lung inflammation induced by lower limb ischemia-reperfusion. If so, then this observation will definitively limit the clinical application of caffeine in this regard. More studies are needed before further conclusion can be reached.

It should be noted that this study confirmed that caffeine exerted significant anti-inflammatory effects in the early

phase of ischemia-reperfusion (i.e., within 6 hours). However, the question of whether caffeine can produce prolonged effects against lower limbs ischemia-reperfusion remains unstudied.

5. Conclusions

Caffeine mitigates lung inflammation induced by lower limbs ischemia-reperfusion in rats.

Disclosure

Part of the study data has been presented at Anesthesiology 2014, the Annual Meeting of the American Society of Anesthesiologists, New Orleans, LA, USA, October 11–15, 2014.

Conflict of Interests

The authors state that no author has personal conflict of interests upon the publication of the material.

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Research Article

3,5,4′-Tri-O-acetylresveratrol Attenuates Lipopolysaccharide-Induced Acute Respiratory Distress Syndrome via MAPK/SIRT1 Pathway

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The aim of the present research was to investigate the protecting effects of 3,5,4′-tri-O-acetylresveratrol (AC-Rsv) on LPS-induced acute respiratory distress syndrome (ARDS). Lung injuries have been evaluated by histological examination, wet-to-dry weight ratios, and cell count and protein content in bronchoalveolar lavage fluid. Inflammation was assessed by MPO activities and cytokine secretion in lungs and cells. The results showed that AC-Rsv significantly reduced the mortality of mice stimulated with LPS. Pretreatment of AC-Rsv attenuated LPS-induced histological changes, alleviated pulmonary edema, reduced blood vascular leakage, and inhibited the MPO activities in lungs. What was more, AC-Rsv and Rsv treatment reduced the secretion of TNF-α, IL-6, and IL-1β in lungs and NR8383 cells, respectively. Further exploration revealed that AC-Rsv and Rsv treatment relieved LPS-induced inhibition on SIRT1 expression and restrained the activation effects of LPS on MAPKs and NF-κB activation both in vitro and in vivo. More importantly, in vivo results have also demonstrated that the protecting effects of Rsv on LPS-induced inflammation would be neutralized when SIRT1 was in-hibited by EX527. Taken together, these results indicated that AC-Rsv protected lung tissue against LPS-induced ARDS by attenuating inflammation via p38 MAPK/SIRT1 pathway.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a common and devastating complication which is mainly caused by direct lung injuries, such as inhalation of toxic substances, and indirect systemic diseases including sepsis and severe trauma. ARDS leads to high morbidity, high mortality, and exorbitant health care costs [1]. Despite adoption of lung-protective ventilation and overall intensive care unit (ICU), the hospital mortality of ARDS patients is still higher than 40% [2]. Sepsis is believed to be one of the most important causes of occurrence and development of ARDS. Numerous researches have declared that inflammation may directly and indirectly affect lung endothelia and the pulmonary hemodynamics by activating inflammatory cells, such as neutrophils, monocytes, and lymphocytes, and proinflammatory mediators, including cytokines, proteases, and cyclooxygenases [3, 4]. Although there is a variety of anti-inflammatory interventions and treatments against the inflammatory process in lungs, the mortality of ARDS remains higher than acceptable [5].

Endotoxin, also known as lipopolysaccharide (LPS), is one of the main ingredients formatting cell wall of Gram-negative bacteria which has also been recognized as the most important pathogen for incurable inflammation. Evidences have revealed that LPS could activate neutrophils, airway epithelial cells, and alveolar macrophages followed by excessive generation of chemokines, such as NO, COX-2, TNF-α, IL-1β, and IL-6. Several studies have addressed that activation of nuclear factor- (NF-) κB and mitogen-activated protein

kinase (MAPK) pathways was responsible for the excessive generation of proinflammatory cytokines [6, 7]. What was more, plenty of evidence indicated that TNF-α and IL-1β may degrade IkBs (IkBα in particular) and translocate NF-κB from the cytoplasm into the nucleus in minutes, which would further amplify the inflammatory process [8]. Besides, overgeneration of inflammatory mediators played a key role in damaging the alveolar-capillary barrier and permeability. Therefore, it may be of great significance to find new ways to interfere with the inflammatory process in treatment of ARDS.

Resveratrol (Rsv) has been known for hundreds of years as powdered root of *Polygonum cuspidatum* and nowadays widely known to exist in grapes, nuts, and red wine [9]. Also, resveratrol is one of the most intensively researched natural compounds since it exhibited protecting effects on multiple diseases, such as cardiovascular disorders [10, 11], cancers [12, 13], and inflammation [14, 15]. There are also plenty of evidences indicating that resveratrol exhibited its pharmacological properties by interfering with the expression and activity of silent information regulator type-1 (SIRT1), which has been demonstrated to play a key role in transcriptional and posttranscriptional regulation of gene expression through the deacetylation of histone and nonhistone proteins [16]. Although resveratrol preserved multiple bioactivities, it has never been adopted as a clinical drug for its poor pharmacokinetic and bioavailability properties [17], while 3,5,4′-tri-O-acetylresveratrol (AC-Rsv), a prodrug of resveratrol firstly reported in 2002 [18], has overcome some of the shortages and results in the accumulation of Rsv in lung [19]. More importantly, researches from our laboratory and other teams have revealed that AC-Rsv attenuated seawater inhalation induced lung injury in rat and reduced γ-irradiation related death in mice [20, 21].

In the present research, we have shown that AC-Rsv exerted protective roles in LPS exposure induced ARDS in mice by modulating SIRT1 expression. Furthermore, our results have demonstrated that the protective effects of AC-Rsv on lung tissue might be through attenuating the pulmonary edema and lung inflammation by inhibiting the p38 mitogen-activated protein kinase (MAPK) pathway.

2. Materials and Methods

2.1. Animals and Agents. Adult male Kunming mice (18–22 g) were obtained from the Animal Center of the Fourth Military Medical University (FMMU, Xi'an, China). The mice were captured in air-filtered, temperature-controlled units with equaled light-dark cycles and had free access to food and water. All experimental processes and treatments to animals have been approved by the Institutional Animal Care and Use Committee of the FMMU according to the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication Number 85-23, revised 1985).

LPS (*Escherichia coli* lipopolysaccharide, 055:B5) and EX527 were obtained from the Sigma Chemical Company (St. Louis, MO, USA). Resveratrol (3,5,4′-trihydroxystilbene) was purchased from Xi'an Grass Plant Technology Corporation (Xi'an, China) with purity above 98%. 3,5,4′-Tri-O-acetylresveratrol (AC-Rsv, structure showed in Figure 1) was synthesized by the Pharmacy Department of Medicinal Chemistry of FMMU with HPLC purity > 99%. Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-6, and IL-1β have been purchased from the R&D Corporation (R&D Systems Inc.). Myeloperoxidase (MPO) activity analyzing kit has been purchased from Jiancheng Bioengineering Institute (Nanjing, China). Antibodies, including anti-p-NF-κB, anti-NF-κB, anti-p-p38 MAPK, anti-p38 MAPK, anti-SIRT1, anti-p-ERK, anti-ERK, and anti-β-actin, have been purchased from the Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

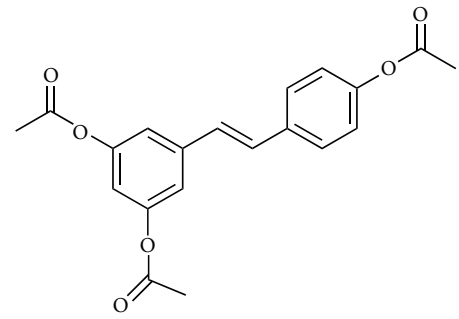

Figure 1: The chemical structure of 3,5,4′-tri-O-acetylresveratrol (AC-Rsv).

2.2. Modeling and Grouping. In order to explore the protecting effects of AC-Rsv on the mortality, mice received an intraperitoneal injection of 20 mg/kg LPS (dissolved in 0.9% saline and filtered through a 0.22 mm membrane) with or without pretreatment of different doses of AC-Rsv (25, 50, or 100 mg/kg body weight) for 7 days. The mortality of mice from different groups ($n = 20$) was recorded every 12 h for 84 h after LPS exposure.

In the research on the protecting effects of AC-Rsv on LPS-induced ARDS, mice were randomly divided into 4 groups: control; LPS (5 mg/kg) only; LPS (5 mg/kg) + AC-Rsv (50 mg/kg); AC-Rsv (50 mg/kg). Mice in the LPS + AC-Rsv group were pretreated with 50 mg/kg AC-Rsv for 7 days and LPS was injected 90 min after the last administration of AC-Rsv since our previous results have indicated that concentration of Rsv in blood reached the peak 90 min after oral administration of AC-Rsv. Mice from all groups were sacrificed 12 h after LPS injection. In addition, the concentration of LPS (20 mg/kg and 5 mg/kg) was selected according to previous researches [22].

2.3. Histological Evaluation. Lung tissues of the same lobe from different groups were fixed with 4% paraformaldehyde for 24 h and then embedded in paraffin. Lung samples were cut into 5 μm thick sections after being deparaffinized and dehydrated. After that, slices of lung samples were stained with hematoxylin and eosin. Finally, all slices were examined and captured under microscope (Leica, Germany).

2.4. Lung Wet-to-Dry Weight Ratios. Lung samples were obtained 12 h after injection of LPS and weighed as soon as possible; after that, all samples were dried to constant weight in an oven at 70°C for 72 h and weighed again. At last, the wet-to-dry weight ratios were calculated by dividing the wet weight of each sample by the dry one.

2.5. Bronchoalveolar Lavage Fluid (BALF) Analysis. At the end of experiments, lungs were removed intactly from mice and lavaged 5 times with 1 mL ice-cold PBS (phosphate buffered saline). The recovery ratio of lavaging fluid was more than 90%. Then, the BALF was centrifuged at 1000 rpm for 5 min at 4°C. The supernatant was collected and protein concentration was determined by BCA method, the results were calculated according to a standard curve, and data were expressed as μg protein per mL (μg/mL). On the other hand, the cell mass was resuspended in red blood cell-lysis buffer and total cell amount was determined by using a hemocytometer.

2.6. Analysis of MPO Activity. MPO activity in lung tissues was evaluated to represent the accumulation of neutrophils in lungs challenged by LPS or not. Briefly, lung samples were homogenized in cold PBS (lung tissue to PBS 1 : 10), and homogenate supernatant was collected by centrifuging at 1000 rpm for 5 min at 4°C. MPO activity was evaluated according to the manufacturer's instructions and values were measured with a spectrophotometer at 460 nm.

2.7. Cell Culture and Treatment. The alveolar macrophage cell line, NR8383, was purchased from the ATCC (USA) and maintained in Ham's F12 medium containing 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

In order to evaluate the protecting effects of Rsv on LPS stimulated NR8383 cells, cells were divided into four groups: control; LPS (1 μg/mL) only; LPS (1 μg/mL) + resveratrol (40 μg/mL); resveratrol (40 μg/mL). Cells from different groups were collected for further investigation after being treated for 12 h. The concentration of LPS in this experiment was selected according to previous researches [22].

In the research of exploring the relationship between SIRT1 expression and the protecting effects of Rsv on LPS stimulated NR8383 cells, cells were divided into four groups and treated with normal Ham's F12 medium (control), LPS (1 μg/mL), LPS (1 μg/mL) + EX527 (1 μM) + resveratrol (40 μg/mL), and LPS (1 μg/mL) + resveratrol (40 μg/mL) for 12 h. Cells were then collected for further examination. By the way, EX527 was first dissolved in dimethyl sulfoxide (DMSO) to 1 mM and then diluted to the working concentration (1 μM) with Ham's F12 medium.

2.8. Measurement of Cytokines. Although it is usually concerning for the distortion of results, ELISA was chosen to analyze the content of TNF-α, IL-6, and IL-1β in lungs and cells in order to provide supplementary data for the current research. Briefly, lung tissues from each group were homogenized in cold PBS (lung tissue to PBS 1 : 5) by using a Tissue-Tearor and cells treated as described above were homogenized by repeated frozen and dissolved method. Homogenates from tissue and cells were centrifuged at 12000 rpm for 5 min at 4°C. Contents of TNF-α, IL-6, and IL-1β in supernatants from tissue and cell samples were measured according to the manufacturer's instructions.

2.9. Western Blot. At the end of the experiment, cell and tissue samples were collected and total proteins were extracted according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China). Protein concentrations were determined by BCA method with an assay kit (Beyotime). After boiling, equal amounts of proteins from each group were separated on SDS-PAGE gel and transferred to polyvinylidene fluoride membranes by wet transfer method. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 followed by incubation with monoclonal antibodies overnight at 4°C against p-NF-κB (1 : 200), p-p38 MAPK (1 : 300), SIRT1 (1 : 200), p-ERK (1 : 200), and β-actin (1 : 5000). Following the incubation of antibodies were repeated washing and incubation of secondary antibody for 2 h at room temperature. Finally, immune complexes were visualized via the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

2.10. Statistical Analysis. Statistical analysis was performed with SPSS 17.0 for Windows. Numeric variables were expressed as means ± S.D. Differences between groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett's test after the distribution of data was confirmed to be a normal distribution. The Kaplan-Meier method and the log-rank test were used to analyze survival data. Statistical significance was accepted as $P < 0.05$.

3. Results

3.1. The Effect of AC-Rsv on LPS-Induced Mortality in Mice. In order to assess the protecting effects of AC-Rsv on endotoxemia, we firstly evaluated the effects of AC-Rsv on LPS-induced mortality in mice. As shown in Figure 2, the accumulative mortalities of mice in middle-dose (50 mg/kg) and high-dose (100 mg/kg) groups were 55% and 65%, respectively, which were significantly lower than that of LPS group (80% mice dead) ($P < 0.05$). Those data indicated that AC-Rsv protected mice from LPS-induced death and 50 mg/kg of AC-Rsv exhibited the best protecting effects which was adopted to carry out further researches.

3.2. Effects of AC-Rsv on LPS-Induced Lung Morphological Changes. Histopathological results showed that lung samples from control (Figure 3(a)) and AC-Rsv (Figure 3(d)) groups exhibited a normal structure with clear pulmonary alveoli, while LPS exposure led to infiltration of inflammatory cells, damage of alveoli, thickened alveolar wall, and formation of hyaline membranes (Figure 3(b)). However, LPS-induced

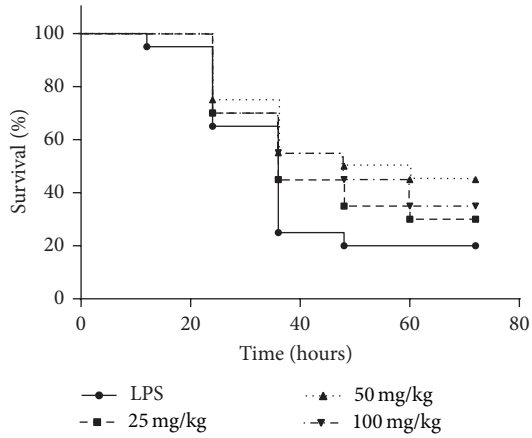

Figure 2: Effects of AC-Rsv on LPS-induced mortality in mice. Mice from each experimental group were injected with 20 mg/kg of LPS with or without pretreatment of AC-Rsv (25, 50, and 100 mg/kg) for 7 days. Alive mice in each group were counted every 12 h. The accumulative mortalities of mice in middle-dose (50 mg/kg) and high-dose (100 mg/kg) groups were 55% and 65%, respectively, which were significantly lower than that of LPS group (80% mice dead) ($P < 0.05$). More importantly, 50 mg/kg of AC-Rsv exhibited the best protecting effect, $P < 0.05$, $n = 20$.

changes in lung structure have been dramatically attenuated by AC-Rsv pretreatment (Figure 3(c)).

3.3. Effects of AC-Rsv on Lung Edema Induced by LPS. The wet-to-dry weight ratios of lung samples from different groups have been calculated in order to assess the pulmonary edema in mice challenged by LPS with or without pretreatment of AC-Rsv. As shown in Figure 3(e), the wet-to-dry ratio was significantly increased in lungs from LPS group compared with that of control ($n = 6$, $P < 0.05$). However, administration of AC-Rsv dramatically reduced water content in lungs 12 h after LPS injection. The result also indicated that AC-Rsv treatment alone did not affect water content in lungs.

3.4. Effects of AC-Rsv on LPS-Induced Lung Vascular Leakage. Cell and protein content in BALF from all four groups were evaluated in order to assess the vascular leakage in lungs stimulated with LPS, as well as determine the protecting effects of AC-Rsv in this respect. As shown in Figures 3(f) and 3(g), LPS injection increased the cell and protein content in BALF compared with those of control ($P < 0.05$), while AC-Rsv restricted cell and protein leakage from pulmonary vessels into the alveoli when stimulated with LPS ($P < 0.05$). Also, there was no significant difference between control and AC-Rsv group.

3.5. Effects of AC-Rsv on Inflammation Induced by LPS in Lungs. In order to evaluate the effects of AC-Rsv on LPS-induced inflammation in lungs, MPO activity (Figure 4) and contents of TNF-α, IL-6, and IL-1β (Figures 5(a)–5(c)) have been measured. The results revealed that LPS injection significantly upregulated the activity of MPO and increased the contents of TNF-α, IL-6, and IL-1β in lungs compared with those of control ($P < 0.05$). However, pretreatment of AC-Rsv dramatically decreased MPO activity and inhibited the concentration of TNF-α, IL-6, and IL-1β in lungs ($P < 0.05$). Also, the results indicated that AC-Rsv treatment alone did not affect the MPO activity and content of cytokines in lungs.

3.6. Effects of AC-Rsv on the Expression of MAPK/SIRT1 Pathway in Lung. In order to further illustrate the mechanisms of how AC-Rsv exhibited the protecting effects against the endotoxemia induced by LPS, the expression of MAPK/SIRT1 pathway was evaluated by western blot. The results (Figure 6) showed that administration of LPS significantly decreased the SIRT1 expression and increased the expression of p-p38 MAPK, p-ERK, and p-NF-κB ($P < 0.05$). However, pretreatment of AC-Rsv dramatically decreased p-p38 MAPK, p-ERK, and p-NF-κB expression and enhanced the expression of SIRT1 in lungs stimulated with LPS ($P < 0.05$).

3.7. Effects of Resveratrol on Generation of Inflammatory Mediators in NR8383 Cells Stimulated by LPS. Based on the findings that AC-Rsv attenuated LPS-induced lung injury, we further evaluated the effects of its intermediate metabolite, resveratrol, on NR8383 cells challenge by LPS because all AC-Rsv would become Rsv in the body [19]. The results (Figures 5(d)–5(f)) showed that LPS stimulation increased the generation of TNF-α, IL-6, and IL-1β in NR8383 cells compared with that of control ($P < 0.05$), while resveratrol significantly reversed this trend and decreased the formation of TNF-α, IL-6, and IL-1β ($P < 0.05$) in NR8383 cells exposed to LPS. What was more, there were no significant differences in the content of chemokine between control and resveratrol groups.

3.8. Effects of Resveratrol on the Expression of MAPK/SIRT1 Pathway in NR8383 Cells. We further evaluated whether resveratrol could modulate the expression of MAPK/SIRT1 pathway in NR8383 cells like AC-Rsv did in LPS challenged lungs. The expressions of SIRT1, p-p38 MAPK, p-ERK, and p-NF-κB have been evaluated in cells stimulated by LPS together with treatment of Rsv or not, and the results (Figure 7) showed that LPS stimulation inhibited the expression of SIRT1 and upregulated p-p38 MAPK, p-ERK, and p-NF-κB expression in NR8383 cells ($P < 0.05$), while resveratrol treatment reversed this trend just like AC-Rsv did in LPS stimulated lungs.

3.9. SIRT1 Inhibitor Neutralized the Protecting Effects of Resveratrol on NR8383 Cells Stimulated by LPS. NR8383 cells were exposed to normal culture, LPS, LPS + EX527 + Rsv, and LPS + Rsv for 12 h, and then the expression of SIRT1 and generation of cytokines were evaluated by western blot and ELISA method, respectively. The results (Figure 8(a)) showed that SIRT1 expression was dramatically suppressed by LPS compared with that of control, while Rsv treatment effectively reversed the decrease of SIRT1 expression. Astonishingly, cotreatment of EX527 and Rsv counterbalanced the elevating effects of Rsv on the expression of SIRT1.

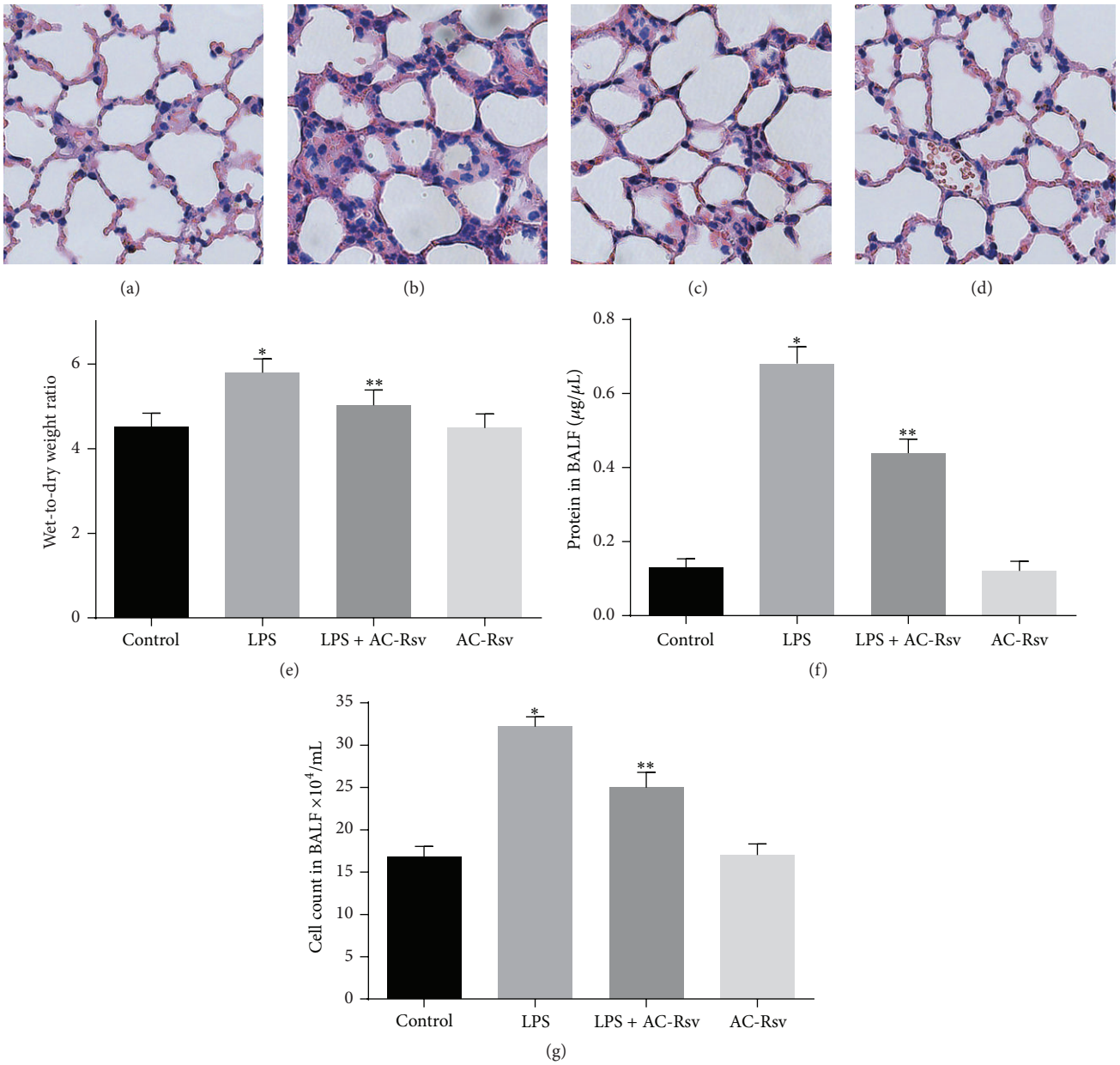

Figure 3: Protecting effects of AC-Rsv on the LPS exposure induced lung injuries. (a–d) Morphological changes were evaluated 12 h after LPS exposure by H&E staining. LPS stimulation group (b) showed increasing lung edema, alveolar hemorrhage, neutrophil infiltration, and destroyed epithelial/endothelial cell structures compared with those of control (a), while significant improvement was observed in samples from the LPS + AC-Rsv group (c). AC-Rsv treatment alone barely affected the structure of lungs (d). (e) Wet-to-dry ratios of lung samples; data are expressed as mean ± S.D. $n = 8$. LPS injection significantly increased the W/D ratios of lung samples compared with that of control, $^{*}P < 0.05$ versus control, while pretreatment of AC-Rsv dramatically decreased the W/D ratios of lung samples stimulated by LPS, $^{**}P < 0.05$ versus $^{*}P$. ((f) and (g)) Protein concentration (a) and cell count (b) in BALF. Data are expressed as mean ± S.D. $n = 8$. LPS exposure significantly increased cell and protein content in BALF compared with those of control, $^{*}P < 0.05$ versus control; and pretreatment of AC-Rsv decreased the cell and protein content in BALF from lung stimulated by LPS, $^{**}P < 0.05$ versus $^{*}P$.

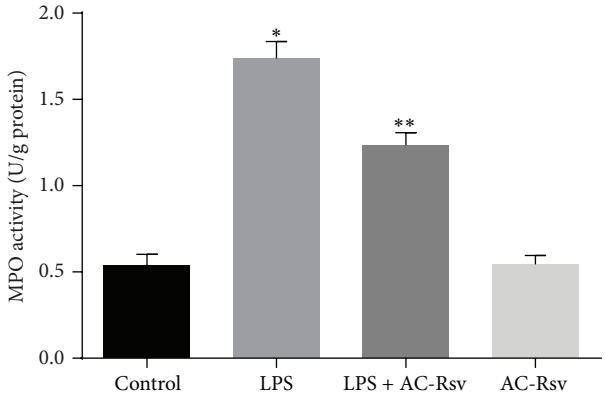

Figure 4: Effects of AC-Rsv on MPO activity in lungs stimulated by LPS. Data are expressed as mean ± S.D. $n = 8$. LPS injection significantly increased the MPO activity in lung compared with that of control, $^{*}P < 0.05$ versus control, while pretreatment of AC-Rsv dramatically inhibited the elevating effects of LPS on MPO activity, $^{**}P < 0.05$ versus $^{*}P$.

On the other hand, the results (Figures 8(b)–8(d)) from the evaluation on the generation of cytokines revealed that LPS stimulation increased the generation of TNF-α, IL-1β, and IL-6, and Rsv treatment reversed this trend. Similar to the effects on SIRT1 expression, coincubation of EX527 and Rsv did not inhibit the generation of those cytokines.

4. Discussion

ARDS is a serious manifestation of systemic inflammation induced by varied factors which may further result in multiple organs dysfunction syndromes (MODS) with a very high mortality [2]. Previous researches have revealed that overactivated inflammatory cells and secretion of inflammatory mediators were the primary pathogenesis for ARDS [23, 24]. There were also researches on the mechanism and treatment of ARDS in vitro and in vivo and substantial progression has been made in understanding of this disease. However, available treatments were still limited in clinic, and it was thus meaningful to explore new pharmacological treatment for ARDS.

As a natural polyphenol, resveratrol (Rsv) has been confirmed to possess a number of pharmacological functions [10–15]. Rsv has never been adopted as a clinical drug for its short half-life, low bioavailability, and poor targeting. However, those shortages have been overcome to some extent by AC-Rsv and administration of AC-Rsv increased the concentration of Rsv in lungs 20-fold compared with that of administration of Rsv [19]. Therefore, we investigated the protecting effects of AC-Rsv on LPS-induced ARDS and the results revealed that AC-Rsv could reduce the mortality rate of mice challenged by LPS and attenuate lung injury by restricting leakage of fluid from blood vessels into pulmonary alveoli, inhibiting the concentration of cytokines and alleviating the abnormal expression of MAPK/SIRT1 expression induced by LPS.

As a hallmark of ARDS [25], pulmonary edema was caused by dysfunction of alveolar-capillary barriers and increased filtration of protein-rich fluid into the alveolar spaces [26]. In the present research, cell content and protein concentration in BALF and lung edema have been measured in order to quantify the magnitude of pulmonary edema and small vascular permeability. The results revealed that administration of LPS increased water content in lung tissues and enhanced infiltration of cells and leakage of protein-rich fluid from blood vessels into alveoli, while pretreatment of AC-Rsv significantly reduced the lung W/D ratio, decreased the protein concentration in BALF, and inhibited the exudation of cells from blood vessels into alveoli induced by LPS.

It has been confirmed that inflammation was another character for ARDS; in addition, neutrophils and macrophages were the main executive cells in LPS-induced pulmonary inflammation [27]. Myeloperoxidase (MPO) was a unique constituent of neutrophil cytoplasmic granules which was indispensable for the killing of phagocytosed pathogens. Therefore, MPO activity was markedly relevant to neutrophils accumulation and served as the marker of inflammation [28]. In the current research, it has been found that MPO activity was dramatically increased in lungs 12 h after LPS exposure, while pretreatment of AC-Rsv significantly decreased the MPO activity. In addition, histopathological study has also revealed that AC-Rsv pretreatment markedly reduced the neutrophil infiltration from blood vessels into pulmonary alveoli.

LPS stimulation as well as activated neutrophils could further induce the generation and secretion of numerous inflammatory mediators and chemotactic cytokines, among which TNF-α, IL-1β, and IL-6 were characterized mediators participating in the occurrence and development of ARDS [29]. Those chemokines were also related to the influx, accumulation, and activation of highly destructive cells involved in inflammation; more importantly, the positive feedback between TNF-α and IL-1β and NF-κB would facilitate more NF-κB expression, phosphorylation, and translocation into nucleus which could further elicit the inflammatory cascade leading to severe damage to lung tissues [30, 31]. In the present research, it has been found that TNF-α, IL-1β, and IL-6 increased remarkably in lungs and NR8383 cells after LPS exposure, while treatment of AC-Rsv and Rsv significantly decreased the generation and secretion of TNF-α, IL-1β, and IL-6 in lung tissues and NR8383 cells, respectively.

In addition to regulating the expression of inflammatory cytokines, AC-Rev should have affected the activation of enzymes and mediators involved in pathogenesis of inflammation. Mitogen-activated protein kinase (MAPK) was a conserved cellular energy status sensor which could be activated by LPS [32, 33]. What was more, activation of MAPKs may further lead to secretion of cytokines such as TNF-α and IL-1β and expression of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) by enhancing transcription of NF-κB [34]. Together with MAPKs, SIRT1 has also been regarded as a key sensor and metabolic modulator for inflammation, especially ARDS. Besides, the corporation between SIRT1 and MAPK in mediating the molecule response to stimuli without or

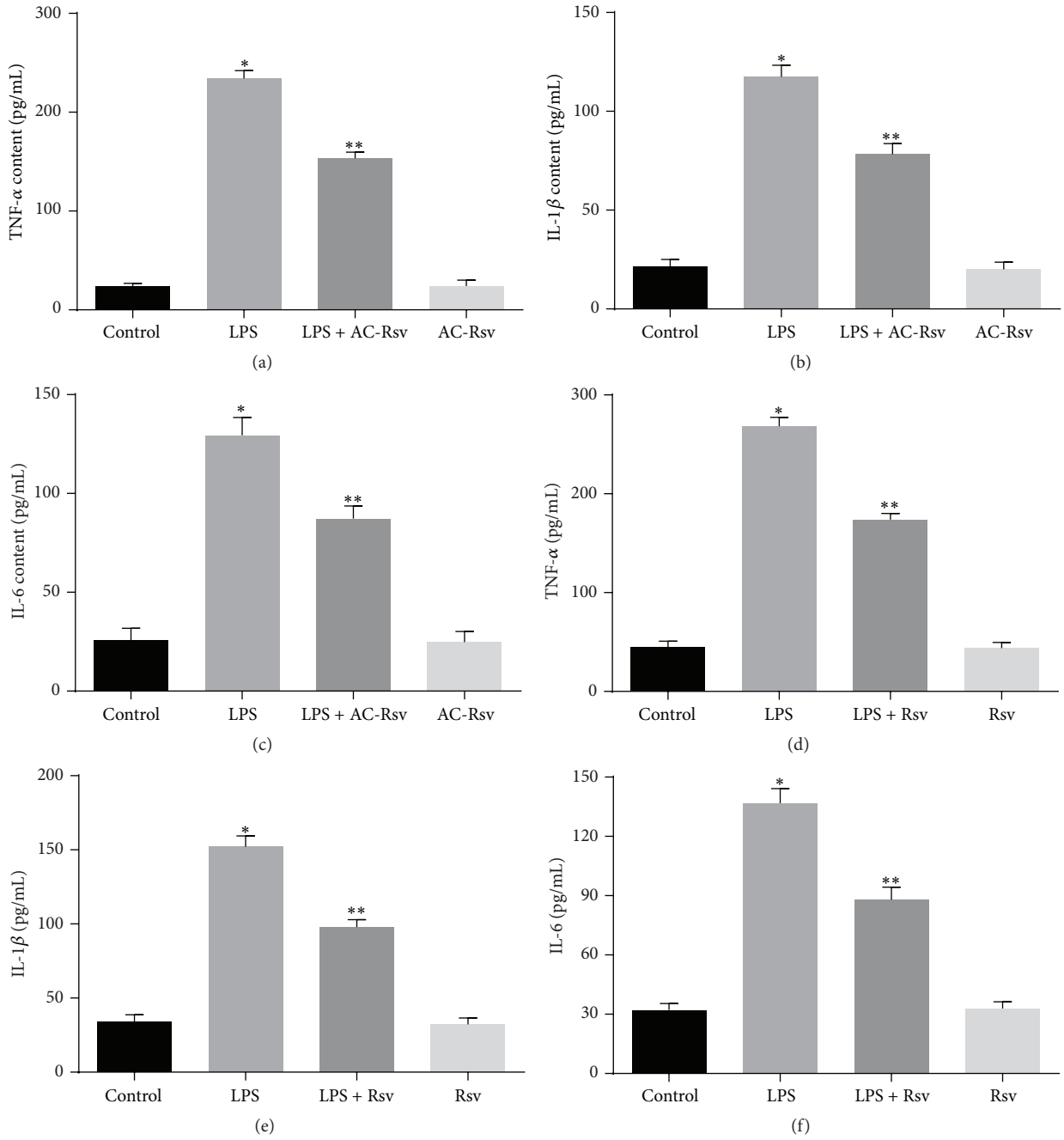

Figure 5: Effects of AC-Rsv on content of cytokines in LPS stimulated lungs and NR8383 cells have been measured by ELISA. (a–c) TNF-α, IL-6, and IL-1β levels in lungs, (d–f) TNF-α, IL-6, and IL-1β levels in NR8383 cells. Data are expressed as mean ± S.D. $n = 8$. LPS stimulation increased the content of TNF-α, IL-6, and IL-1β in lungs and NR8383 cells compared with that of control, $^{*}P < 0.05$ versus control, while AC-Rsv and Rsv treatment significantly inhibited the formation of those cytokines in lung and NR8383 cells, respectively. $^{**}P < 0.05$ versus $^{*}P$.

within pretreatment was closely related to NF-κB [35, 36]. Based on those clues, we explored the expression of SIRT1 and MAPK pathway in LPS-induced ARDS; the results showed that LPS exposure inhibited SIRT1 expression and enhanced the phosphorylation of p38 MAPK and ERK followed by increased p-NF-κB expression in vitro and in vivo. However, pretreatment of AC-Rsv relieved LPS-induced inhibition on SIRT1 expression and restrained the effects of LPS on p38 MAPK, ERK, and NF-κB in lungs and NR8383 cells.

To further confirm the protective role of AC-Rsv on LPS-induced ARDS via interfering with the expression of SIRT1, in vitro experiment has been carried out by cotreatment of EX527 and Rsv for LPS stimulated NR8383 cells. EX527 is a new type of highly selective inhibitor for SIRT1 which

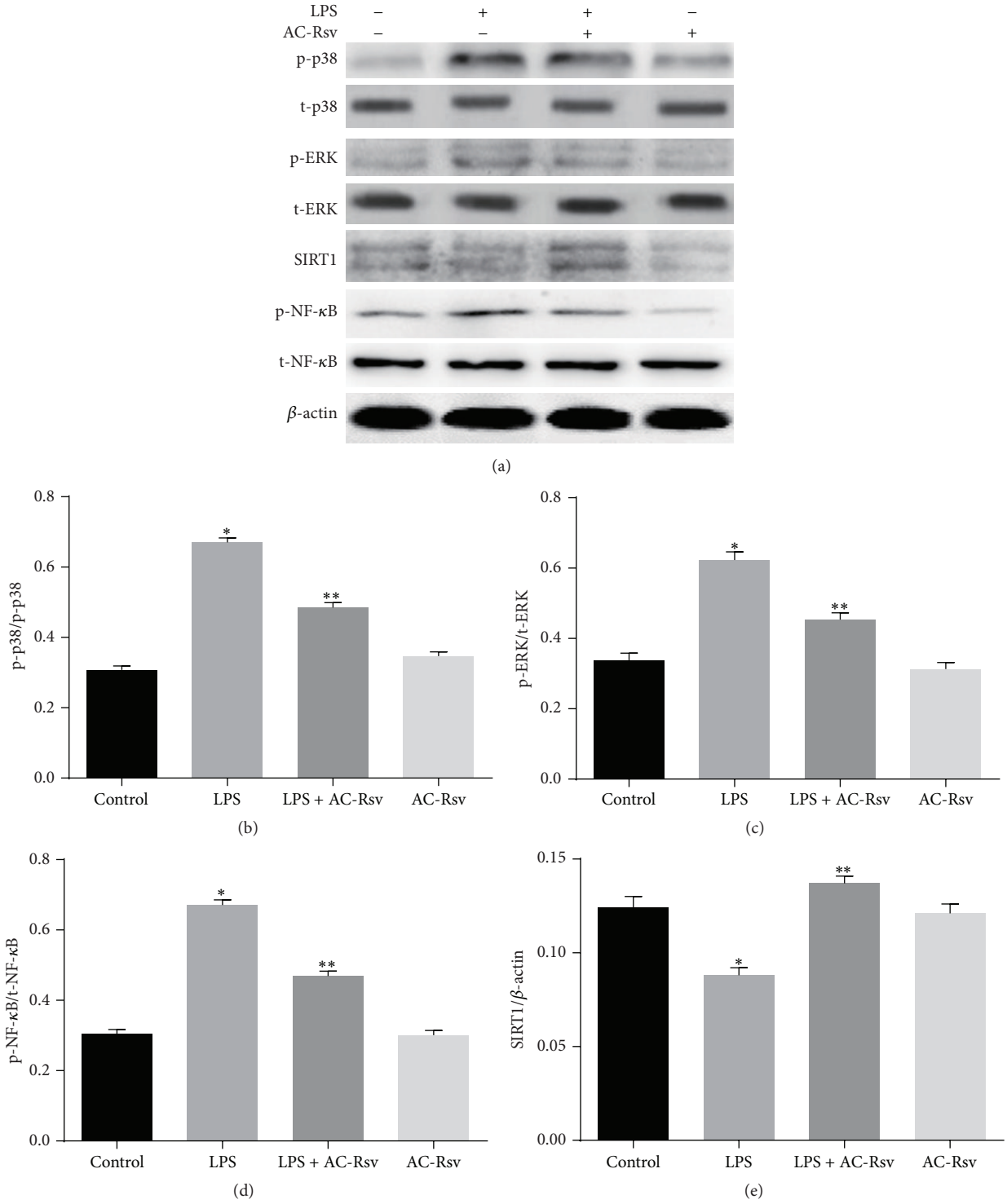

Figure 6: Effects of AC-Rsv on the expression of MAPK/SIRT1 axis in lungs were evaluated by western blot. Relative expression levels of p-p38 MAPK, p-ERK, and p-NF-κB were normalized to the expression levels of their total forms; SIRT1 expression was expressed by comparing with that of β-actin. Administration of LPS significantly decreased the SIRT1 expression and increased the expression of p-p38 MAPK, p-ERK, and p-NF-κB in lungs compared with that of control, $^{*}P < 0.05$ versus control, while AC-Rsv treatment dramatically decreased expression of p-p38 MAPK, p-ERK, and p-NF-κB and enhanced the SIRT1 expression in lungs when challenged by LPS. $^{**}P < 0.05$ versus $^{*}P$.

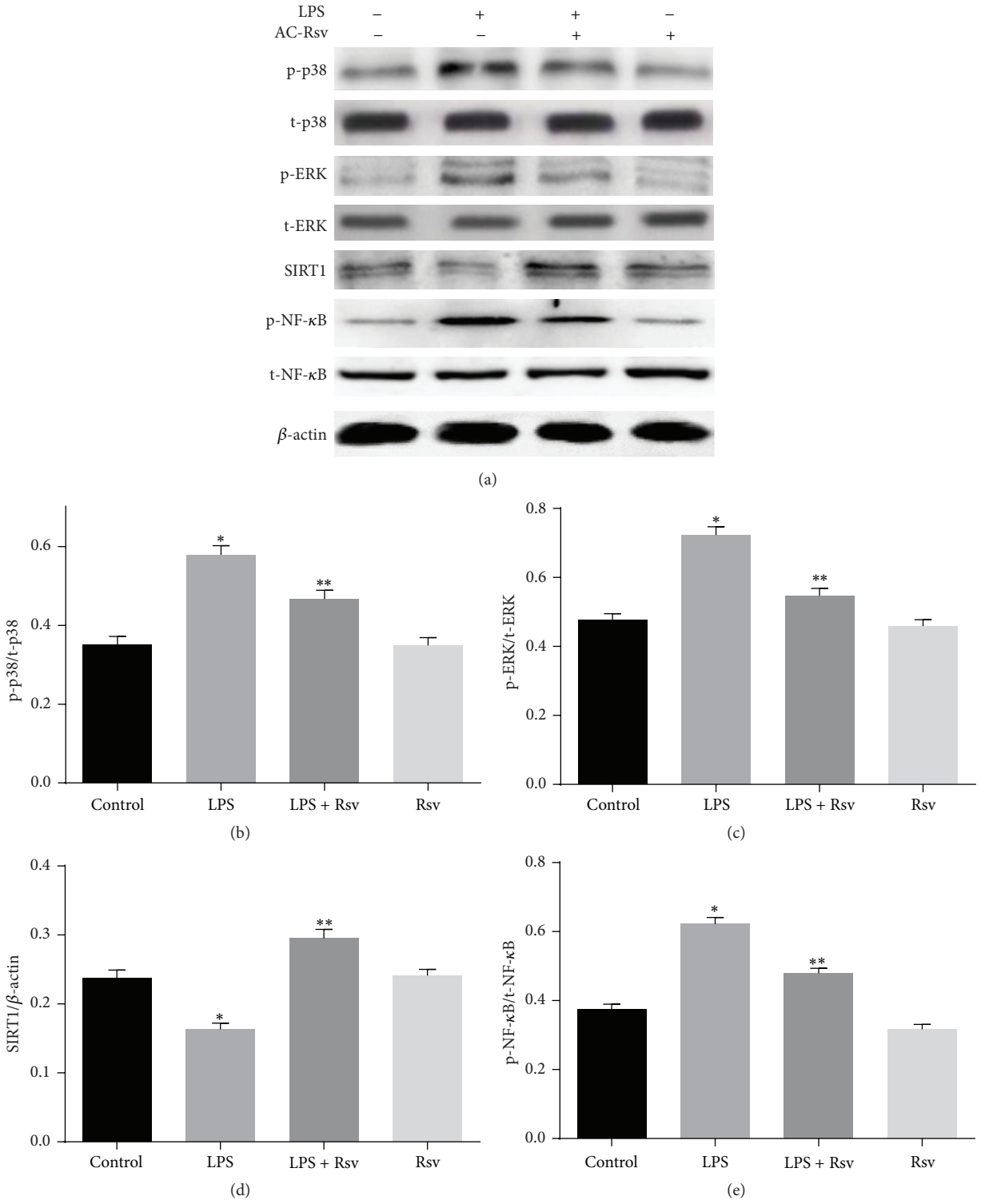

Figure 7: Effects of Rsv on the expression of MAPK/SIRT1 pathway in NR8383 cells stimulated by LPS. Relative expression levels of p-p38 MAPK, p-ERK, and p-NF-κB were normalized to the expression levels of their total forms; SIRT1 expression was expressed by comparing with that of β-actin. Stimulation of LPS significantly decreased the SIRT1 expression and increased the expression of p-p38 MAPK, p-ERK, and p-NF-κB in NR8383 cells compared with that of control, $^{*}P < 0.05$ versus control, while Rsv treatment dramatically decreased p-p38 MAPK, p-ERK, and p-NF-κB expression and enhanced the expression of SIRT1 in NR8383 cells when challenged by LPS. $^{**}P < 0.05$ versus $^{*}P$.

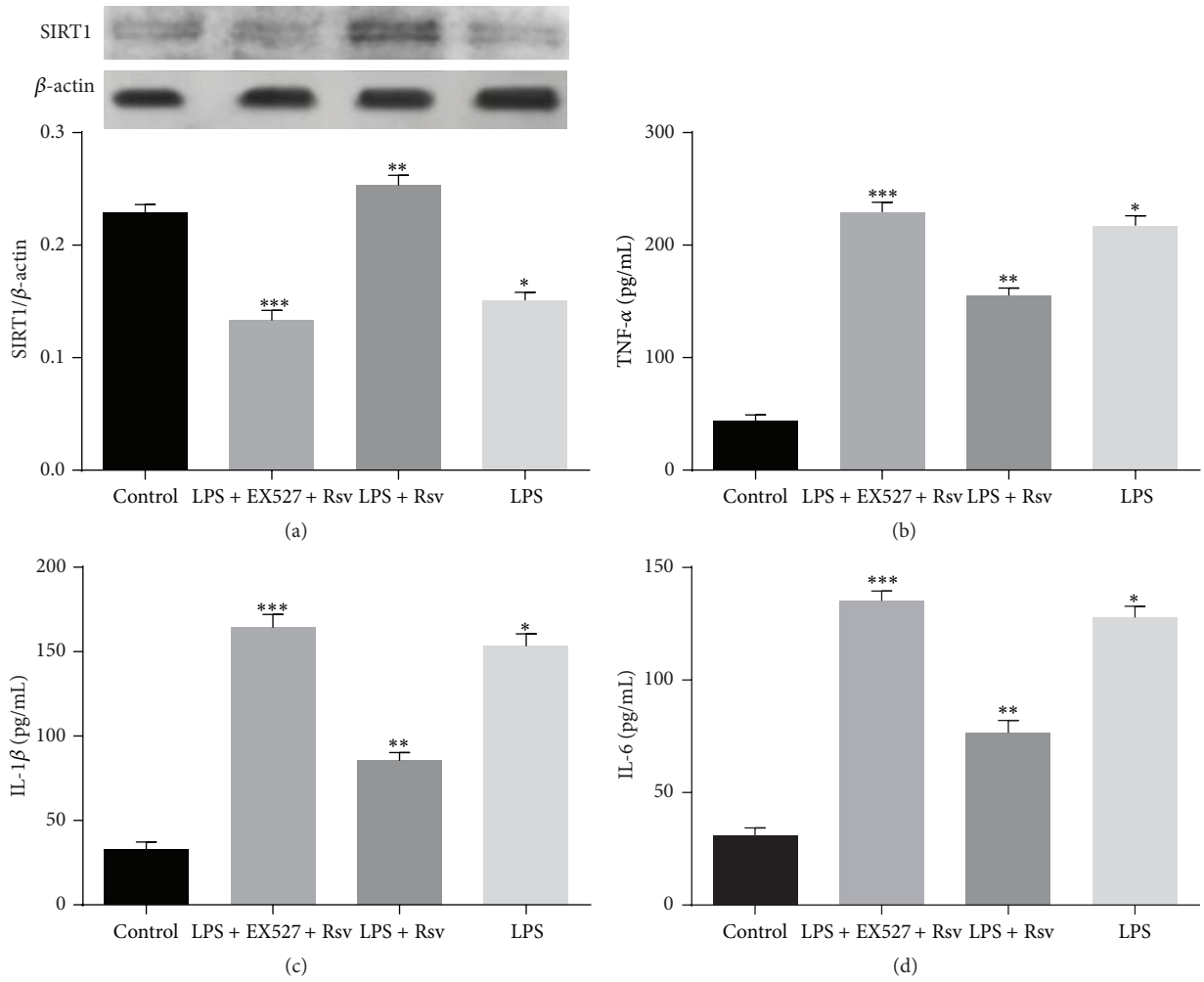

Figure 8: Neutralizing effects of EX527 on the protecting effects of Rsv on LPS stimulated NR8383 cells. (a) Expression of SIRT1 was evaluated by western blot and normalized to the expression level of β-actin. LPS stimulation inhibited the expression of SIRT1 compared with that of control, $^{*}P < 0.05$ versus control, while Rsv treatment significantly reversed the inhibiting effects, $^{**}P < 0.05$ versus $^{*}P$; on the contrary, EX527 and Rsv cotreatment counterbalanced the enhancing effects of Rsv on the expression of SIRT1, $^{***}P < 0.05$ versus $^{**}P$. (b–d) Contents of TNF-α, IL-6, and IL-1β were evaluated by ELISA. LPS stimulation increased the generation of TNF-α, IL-6, and IL-1β in NR8383 cells compared with that of control, $^{*}P < 0.05$ versus control, while Rsv treatment significantly inhibited the formation of those cytokines in NR8383 cells when stimulated by LPS, $^{**}P < 0.05$ versus $^{*}P$; astonishingly, EX527 and Rsv cotreatment neutralized the inhibiting effects of Rsv on the generation of TNF-α, IL-6, and IL-1β. $^{***}P < 0.05$ versus $^{**}P$.

could effectively inhibit the acetylation enzyme activity of SIRT1 (IC_{50} 38 nmol/L) [37]. As expected, LPS stimulation decreased SIRT1 expression and increased the generation of TNF-α, IL-1β, and IL-6, while Rsv treatment could reverse this trend to some extent. However, cotreatment of EX527 and Rsv counterbalanced the attenuating effects of Rsv on decreased expression of SIRT1 and accelerated generation of TNF-α, IL-1β, and IL-6 resulting from LPS stimulation. Taken together, those results indicated that the activation of SIRT1 by Rsv attenuated the inflammation in NR8383 cells stimulated by LPS, while the inhibition of SIRT1 by EX527 could neutralize the protecting effects of Rsv on LPS-induced inflammation and lung injury.

In summary, results from the present research indicated that AC-Rsv pretreatment therapeutically attenuated LPS-induced ARDS by suppressing lung edema, inhibiting leakage of protein-rich fluid from blood vessels into pulmonary alveoli, and depressing inflammatory process in mice. The mechanisms underlying the therapeutic effects of AC-Rsv were probably via the MAPK/SIRT1 pathways. Although further researches were needed, the present research indicated that AC-Rsv may be a potential therapeutic agent for ARDS.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Lijie Ma and Yilin Zhao contributed to the paper equally.

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Review Article

Acute Respiratory Distress Syndrome: Role of Oleic Acid-Triggered Lung Injury and Inflammation

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Lung injury especially acute respiratory distress syndrome (ARDS) can be triggered by diverse stimuli, including fatty acids and microbes. ARDS affects thousands of people worldwide each year, presenting high mortality rate and having an economic impact. One of the hallmarks of lung injury is edema formation with alveoli flooding. Animal models are used to study lung injury. Oleic acid-induced lung injury is a widely used model resembling the human disease. The oleic acid has been linked to metabolic and inflammatory diseases; here we focus on lung injury. Firstly, we briefly discuss ARDS and secondly we address the mechanisms by which oleic acid triggers lung injury and inflammation.

1. Introduction

Despite advances in the past decades in the knowledge and treatment of acute respiratory distress syndrome (ARDS) the mortality remains unacceptably high [1], ranging from 27% to 45% [2]. Here we discuss lung injury and inflammation mechanisms induced by a single fatty acid molecule, the oleic acid. For that, we focus on mechanisms of cell death, edema formation and alveoli swelling, cell and intracellular signaling activation pathways, and inflammatory mediator production that would lead to severe lung damage and loss of function.

2. Acute Respiratory Distress Syndrome

The lung is the primary target of diverse insults including, but not limited to, microbe's infection, pollutants, toxic gasses, gastric acids, autoantibodies, fatty emboli, and free fatty acids (Table 1). Initial lung injury can evolve to a severe disease known as acute respiratory distress syndrome (ARDS) [3]. One of the initial steps of this syndrome is the edema formation followed by an intense inflammatory response causing lung functional and structural damage and patients with ARDS demand intensive care treatment.

The epithelial cell lining in the alveoli-endothelial barrier produces a thin liquid layer containing secreted peptides and proteins contributing to host defense and preserving the primary lung function of transporting CO_2 from the blood to alveoli and O_2 from the alveoli to the bloodstream (Table 2). Alveoli are the functional lung unit, and they are covered by alveolar type I and type II cells (Figure 1). Alveolar type I cells comprise approximately 90% of the alveolar epithelium, and the remaining 10% is formed by cuboidal type II cells that handle surfactant secretion and by epithelial cell regeneration after injuries (Table 2).

Lesions to the alveolar capillary endothelium and epithelium result in barrier disruption leading to plasma proteins leakage and edema formation characterizing the exudative phase [4]. In addition to activation of the other effectors cells such as neutrophils, macrophages, endothelium, and epithelium activation, platelets also contribute to the alveolar

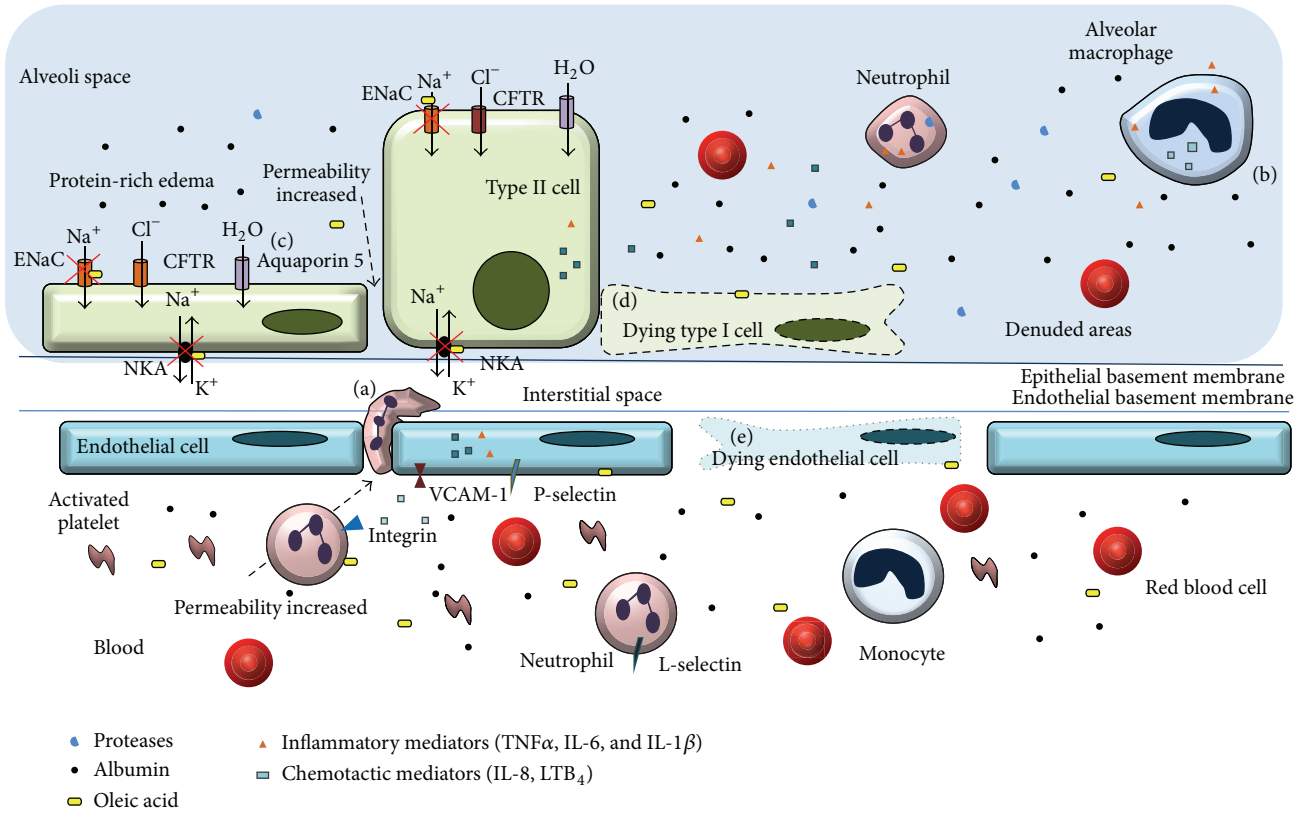

Figure 1: Effects of oleic acid in acute respiratory distress syndrome. Oleic acid induces damage in epithelial and endothelial cells, with increased permeability and protein-rich edema, with denuded areas in alveoli forming a hyaline membrane. Oleic acid induces apoptosis or necrosis in alveolar type I or type II cells (d), depending on the insult origin. Alveolar macrophages (b) act as sentinels triggering the immune response and produce chemotactic and inflammatory mediators. Chemoattractant mediators produced by alveolar macrophages and epithelial and endothelial cells induce increased adhesion molecules such as VCAM-1, selectins, and integrins, favoring the inflammatory cell infiltration. Neutrophils (a) are first cells migrating to lung and their excessive recruitment contributes to the lung pathology and they produce and release other inflammatory mediators and other molecules such as proteases and elastase. Aquaporin 5 is (c) a water channel responsible for moving water from the alveoli to the lung interstitium, AO-induced lung injury could advent via intrapulmonary or extrapulmonary. In case of extrapulmonary ARDS the main target will be endothelial cells and leukocytes inducing endothelial cell death (e). Similar to humans, OA induces lung hemorrhage. OA inhibits ENaC and NKA inducing and/or avoiding edema fluid clearance. ENaC: epithelial sodium channel; CFTR: cystic fibrosis transmembrane conductance regulator; NKA: Na/K-ATPase; VCAM-1: vascular cell adhesion molecule 1.

damage in lung injury [3]. In the acute phase, cytokines and lipids are released, leading to alveolar-capillary barrier loss and hyaline membrane formation [5].

ARDS is a syndrome comprising respiratory failure with acute hypoxemia and alveolar damage secondary to an intense lung inflammatory response to different types of insults which is not mainly due to left atrial hypertension [6]. Recently, a new definition (the Berlin definition) proposed 3 mutually exclusive categories of ARDS based on the degree of hypoxemia, mild, moderate, and severe [2], and, therefore, the term acute lung injury (ALI) is no longer used.

Table 1: Major agents that cause pulmonary injury.

Cell type	Agents
Alveolar types I and II cells	Pulmonary aspiration (HCl), trauma, lung infection (alive microbes or microbes secreted molecules), smoke inhalation (tobacco and other molecules), oleic acid, LPS, drug overdose, and inflammatory mediators
Endothelial cells	Systemic infection (sepsis, alive microbes or metabolic products), oleic acid, LPS, fatty embolism, large volume blood replacement, burn injury, inflammatory mediators, and autoantibodies

3. Inflammatory Cells and Mediators

The incidence of leukocytes together with alveolar edema, hemorrhage, and hyaline membrane formation indicates that an exaggerated inflammatory response underlies the pathogenesis of early steps of pulmonary ARDS. Recognition of danger-associated molecular patterns (DAMP) by lung epithelium and alveolar macrophages is a compelling force to induce acute lung inflammation [7] (Table 2). The unbalanced inflammatory response including leukocytes recruitment and/or their activation may damage the epithelial or

Table 2: Functions of the main lung cell types affected in ARDS-lung injury.

Cell type	Functions
Alveolar type I cell	Majority of the alveolar surface coverage, alveolar-capillary barrier formation, alveolar fluid clearance, and gas exchange
Alveolar type II cell	Surfactant secretion, epithelial cell regeneration after injuries, alveolar-capillary barrier formation, alveolar fluid clearance, gas exchange, and inflammatory mediators formation
Endothelial cell	Alveolar-capillary barrier formation, gas exchange, and inflammatory mediators production
Alveolar macrophages	Danger-associated molecular patterns recognition, immune response triggering, and chemotactic and inflammatory mediators secretion

endothelial layer. Neutrophils are first cells migrating to lung and their excessive recruitment contributes to the tissue damage and inflammation [8] (Figure 1(a)) because they release proteases and increase the production of reactive oxygen species and inflammatory mediators [3]. In mice, key chemotactic factors to neutrophil recruitment to the lung are the chemokine CXCL1/GRO alpha (also known as KC) and chemokine (C-X-C motif) ligand 2 (CXCL2) and CXCL5 [9]. Extracellular ATP (eATP) also plays a role in neutrophil recruitment [10]. Alveolar macrophages (Figure 1(b)) act as sentinels triggering the immune response and producing chemotactic and inflammatory mediators [3]. Thrombospondin-1, a circulating plasma glycoprotein detected in bronchoalveolar lavage fluids in ARDS patients, disrupts the endothelial barrier by tyrosine kinase-dependent phosphorylation of zonula adherens proteins [11].

Cytokines such as TNFα and interleukins (mainly IL-1β and IL-6) are important mediators in the development of ARDS, contributing to augmented vascular permeability and organ dysfunction [12]. High pulmonary edema fluid levels induced by IL-8 were associated with impaired alveolar fluid clearance in ARDS patients [13].

4. Lung Edema Clearance

Pulmonary edema results from a combination of both increased fluid filtration and impairment of transepithelial Na^+ transport. Alveolar fluid clearance (AFC) is driven by sodium transport across the airway epithelium, which creates mini-osmotic gradient removing water from the alveoli driving it to the bloodstream.

This mechanism depends on the apically located epithelial sodium channel (ENaC) and the basolaterally located enzyme sodium potassium ATPase (NKA). β-adrenergic agonists are noticeable activators of Na^+ channels in the alveolar epithelium improving fluid clearance and edema resolution in experimental ARDS models [14]. Moreover, impairment of the enzyme NKA during ARDS not only avoids the resolution of lung edema but also intensifies its formation. In this regard, Na^+ transport and edema clearance are associated with better outcomes in patients with sepsis and ARDS [11].

In addition to the sodium transport, the chloride transport via the cystic fibrosis transmembrane conductance regulator (CFTR) is necessary for the AFC. In an animal model of cystic fibrosis, with the lack of cystic fibrosis transmembrane conductance regulator (CFTR), alveolar fluid clearance was decreased. Using glibenclamide (an inhibitor of potassium and CFTR channels) in *in situ* perfused and nonperfused mouse lungs and in *ex vivo* human lungs, fluid clearance was impaired [11].

Finally, water crosses the alveolar epithelium either paracellularly via tight junctions or transcellularly via aquaporins [14]. Aquaporin 5 (AQP5) is expressed on the apical surface of both cell types I and II (Figure 1(c)) and is responsible for moving water from the alveoli to the lung interstitium. A significant decrease in airway-capillary water permeability is seen in lungs of AQP5 deficient mice [15].

Besides alveolar liquid, protein excess needs to be removed from alveolar space, albumin can be taken up by alveolar epithelial cells by the multiligand receptor megalin (low-density lipoprotein endocytic receptor family), and its inhibition resulted in decreased albumin binding and uptake in monolayers of primary alveolar type II and type I cells in cultured lung cells [16].

Overall, the rate of alveolar fluid transport depends on the expression and activity of ENaC, NKA, and CFTR opening. To complete edema reabsorption ion transport, water channels, and albumin transport are also important. Therefore, endothelial and epithelial barrier integrity is essential for optimal fluid balance and cell injury and/or defects on the ion transport caused by pathogens or other damaging compounds end up in decreased AFC [6]. The lesser AFC correlates with a longer stay in the intensive care unit and increased mortality in patients.

5. Origin of Pulmonary Insult

The ARDS pathogenesis has been classified as pulmonary (with a direct hit on lung cells) or extrapulmonary (with an indirect hit, affecting a distant organ and leading to a systemic inflammatory response) [17]. Despite the insult applied to the lung, through airways or circulation, the final result is diffuse alveolar damage. Then, any local (e.g., pneumonia) or systemic inflammation (e.g., pancreatitis) can lead to critical lung function alterations. An extensive injury to the epithelial and endothelial cell, hyaline membrane formation, and increased amount of apoptotic neutrophils is observed in pulmonary insult. In the extrapulmonary injury mediators released from extrapulmonary locations into the blood target mainly endothelial cells, leading to microvascular congestion, endothelial cell activation, an increase in vascular permeability, and interstitial edema [18].

6. Oleic Acid

Oleic acid (18:1 n-9) is an unsaturated fatty acid in plants and animals [19, 20]. Oleic acid is the most common and abundant fatty acid in the body of healthy individuals. It is

Table 3: Key features of oleic acid-triggered lung injury and inflammation.

	Oleic acid
Direct and indirect lung injury induction	x
Cytokine induction	TNFα, IL-6, and IL-1β
Chemokine induction	IL-8, MIP-1α
Cell death induction	Apoptosis, necrosis
Sodium potassium ATPase inhibition	x
Immune innate response receptor activation	GPRC, NKA signalosome
Hyaline membrane formation	x
Lung hemorrhage induction	x
Lung cell infiltration/accumulation	Neutrophil, mononuclear cells
Lung function impairment	x
Protein-rich edema formation	x
Time line, course of lung injury	5 min up to 24 h
Lipid body formation	x
Lipid mediator induction	PGE_2, LTB_4
Intracellular pathway activation	MAPK ERK1/2, PI3K/Akt, sPLA(2), caspases 3 and 6, apelin-13, and mTOR

present in human plasma, cell membranes, and adipose tissue [21, 22]. Oleic acid not only affects membrane fluidity but also facilitates membrane docking and activity of G-protein coupled receptors (GPCR) and related signaling molecules [21].

The effects of oleic acid on cells are mediated by mechanisms such as signaling through cell surface receptors or nuclear receptors [23]. Free fatty acid receptor 1 and GPR120 are membrane GPCR activated by medium and long-chain free fatty acids, as oleic acid. Free fatty acid receptor 1 also known as FFAR1 or GPR40 [24] is expressed primarily in pancreatic beta-cells and contributes to insulin secretion. Its activation leads to an increase in the intracellular Ca^{++} concentration and activation of the extracellular signal-regulated kinase (ERK)1/2 in CHO cells [23] (Table 3).

Nonesterified fatty acids (NEFA) are carried in the bloodstream bound to albumin, thus avoiding their cytotoxicity [25]. Different cells exhibit morphological features of apoptosis and necrosis after fatty acid exposure [26]. Fatty acids also alter the membrane structure, transmembrane signaling, and cell cycle control [27, 28]. They can also modify cellular functions requiring the participation of peroxisome proliferators-activated receptors (PPAR). PPAR are nuclear receptors that regulate the lipid metabolism, inflammation, cellular growth, and differentiation [29].

Altered circulating fatty acid levels are linked to pathologies such as obesity, diabetes mellitus, coronary heart disease, atherosclerosis, and cancer [30]. More important, the severity of diseases such as sepsis, leptospirosis, pancreatitis, and preeclampsia correlates with increased serum fatty acids levels and the drop in plasma albumin concentration, suggesting fatty acid toxicity [31–33].

7. Oleic Acid-Induced Lung Injury and Inflammation

Oleic acid lung injury presents an early phase of necrosis and microvascular thrombosis, followed by a repair phase with the proliferation of type II cells and fibrotic foci in subpleural areas [34]. Microscopically, the injury is multifocal and heterogeneous, ranging from small edema areas to hemorrhagic infiltration with fibrin deposition [35]. The histological changes of oleic acid-induced lung injury are associated with marked functional changes. As in ARDS, lung injury after oleic acid challenge presented extravasation of fluid to the extravascular space and decreased liquid reabsorption, resulting in extravascular lung water accumulation. Pulmonary microvascular permeability is markedly increased, with extravascular lung water accumulation and leakage of protein-rich fluid into the air spaces [36] (Figure 1(d)).

The foremost target organ after oleic acid intravenous inoculation is the lungs, which retains about 85% of free fatty acids. The initial lesions occur as early as 5 min after administration [37] and last at least for 24 hours [38]. Oleic acid injected into the lung also induces neutrophil accumulation [36]. Increased TNFα and IL-8 levels after oleic acid injection [39], as well as IL-6, IL-1β, and the chemokine MIP-1α [36], were reported. Hence, oleic acid induces the synthesis of the main inflammatory mediators involved in clinical ARDS (Table 3 and Figures 1 and 2).

Lipid bodies numbers are increased in cells involved in inflammatory and immunologic processes [40]. Lipid bodies function as privileged sites is generating the lipid mediators LTB_4 and PGE_2 [41]. Oleic acid intratracheal instillation augmented lipid body numbers and LTB_4 [42] and PGE_2 levels [42, 43]. The intravenous injection of oleic acid increased lipid bodies formation and increased PGE_2 levels in bronchoalveolar fluid lavage (BALF) [38] (Table 3 and Figure 1). Remarkably, the rise of LTB_4 and PGE_2 in human samples preceded ARDS in injured blunt-trauma patients [44], indicating similar features between experimental models using oleic acid and clinical events. Also, similar to oleic acid-induced lung injury, hemorrhage can arise in severe ARDS seen in patients with complicated leptospirosis [36, 45] (Figure 1).

Pulmonary edema formation can represent a life-threatening situation if it is not properly removed. Oleic acid is a NKA inhibitor [46] and also a Na^+ channel inhibitor in the lung [47] resulting in a significantly increased endothelial permeability. We developed an assay that may allow researchers to study the importance of NKA activity using OA and ouabain (a classical NKA inhibitor) as a prove-of-concept control [48] (Figure 1). We showed that oleic acid inhibited NKA *in vivo* by measuring the uptake of rubidium by lung tissue and further that oleic acid inhibition was similar to ouabain. This animal model can be used to assay NKA inhibition not only in oleic acid-induced lung injury but also when using other molecules [48].

The leptospiral component glycolipoprotein fraction (GLP) has cytotoxic activity, and oleic acid is a major component of GLP [49]. Furthermore, we showed that the GLP lipid content handles NKA inhibition indicating that oleic acid has a crucial role in NKA inhibition either alone or as a part of

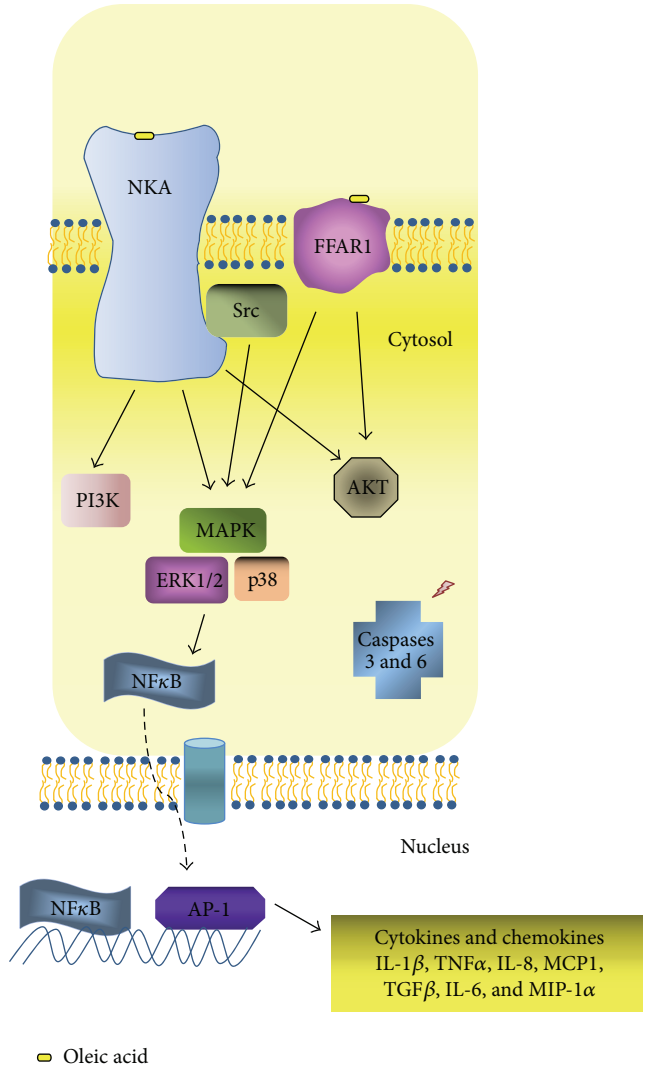

Figure 2: Intracellular pathways activated in oleic acid-induced lung injury and inflammation. Oleic acid triggers intracellular pathways through different receptors ending up in inflammatory mediator production and/or cell death. MAPK: mitogen-activated protein kinases, ERK1/2: extracellular signal-regulated kinases, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells, PI3K: phosphatidylinositol 3-kinase, AKT: protein kinase B, NLRC4: NLR family CARD domain-containing protein 4, MyD88: myeloid differentiation primary response gene 88, AP-1: activator protein 1, TLR: toll-like receptor, IL: interleukin, MIP: macrophage inflammatory protein, FFAR1: free fatty acid receptor 1, MCP1: monocyte chemotactic protein 1, TGFβ: transforming growth factor beta, and TNFα: tumor necrosis factor alpha.

a macromolecular complex. Recently we showed that GLP induces lung injury similar to ouabain and oleic acid [50]. Thus, oleic acid prevents edema clearance and can trigger protein-rich edema formation by intravenous or intratracheal routes [36, 38].

Intracellular Pathways Activated in Oleic Acid-Induced Lung Injury and Inflammation. Oleic acid may trigger diverse intracellular pathways altering cell functions. Here we discuss critical pathways induced by oleic acid impacting on lung damage.

The protein phosphatase and tensin homologue deleted on chromosome Ten (PTEN) is a major suppressor of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling, a vital survival pathway in lung cells (Figure 2). PTEN inhibition by bpV(phen) increased lung tissue levels of phospho-Akt and ERK and reduced the severity of oleic acid-induced ARDS in mice [51] (Table 3). ERK pathway participates in chemoattractant-induced neutrophil chemotaxis and respiratory burst as well as in LPS-induced ARDS [52]. In alveolar macrophages, the combined inhibition of p38 and ERK1/2 induced suppression of cytokine release [53]. ERK1/2 inhibition blocked neutrophil migration, edema, lipid body formation, and IL-6 production in a mice model of oleic acid-induced lung injury [36].

The serine/threonine kinase mammalian target of rapamycin (mTOR) is a key signaling kinase linked to several cellular functions including immunological and inflammatory responses. The mTOR inhibition reduced inflammatory cytokines in LPS/oleic acid-induced lung injury model [54].

Apelin is a group of small peptides derived from a common precursor, preproapelin. All apelin peptides exert their biologic effects by binding to a G-protein-coupled receptor, the APJ receptor, leading to biologic responses [55]. The apelin and APJ receptor are upregulated during tissue injury [56, 57]. A recent report showed that the inhibition of apelin-APJ alleviated lung inflammation and injury and improved oxygenation in oleic acid-induced lung injury [58].

Cell damage caused by the direct binding of oleic acid to biological membranes may be pivotal in oleic acid-mediated lung injury. Oleic acid triggers intracellular pathways ending up in lung cells death. It is directly toxic to endothelial cells in the lung [37], causing necrosis and inducing capillary congestion and interstitial/intra-alveolar edema [35] (Figure 1). Oleic acid induces mainly necrosis, but it also provokes apoptosis through a decrease in the antiapoptotic marker Bcl-2 and a marked increase of proapoptotic marker Bad [59]. Oleic acid also activates caspases 3 and 6 (Figure 2), enhancing the generation of reactive oxygen species and inducing a significant mitochondrial depolarization and apoptosis in leukocytes [60–62].

Oleic acid may work as a particular chaperon that modulates the interaction of cardiac glycosides with the NKA and hence alteration of oleic acid plasma levels would alter ouabain effects [63]. The NKA interacts with different signaling proteins forming a protein complex called signalosome [64]. The NKA signal transduction and ion pump functions work in independent fashion [65]. The oleic acid-induced lung inflammation may start with NKA activation [38]. Oleic acid triggers intracellular pathways in the lung and here we reinforce the critical role of NKA for inflammation in oleic acid-induced lung injury.

8. Animal Models of Oleic Acid-Induced Lung Injury

Animal models of lung injury were developed in the attempt to mimic the human ARDS. ARDS animal models may help us to understand the mechanism of ARDS. Unfortunately, no animal model mimics human ARDS exactly. Nonetheless, animal's studies can bring up crucial elements of lung injury in humans. In this regard, animal models can serve as a bridge between patients and lab research.

The oleic acid model was developed as an attempt to reproduce ARDS due to lipid embolism [66]. Variation in the outcome of this model is likely a consequence of variation in the preparation of oleic acid infusion that could be avoided by injection of oleic acid in a salt form [42], avoiding unwanted effects of ethanol, DMSO, or fatty embolism caused during blood emulsification.

The oleic acid induces early, fast, and reversible sparse inflammatory lung injury with permeability alterations and deficiency in gas exchange and lung mechanics. One advantage of this model is its reproducibility. The oleic acid inoculation provides a superb model to study ventilatory strategies, lung mechanics, and ventilation/perfusion ratio distribution during lung injury in large and small animals [35, 39, 67].

One drawback is the requirement of expertise in intravenous administration in small animals like mice. Another possible downside of the model is the relevance in human disease of oleic acid-induced ARDS. Here we strongly advocate in favor of a high relevance lipid metabolism alteration linked to ARDS: in particular, but not limited to, cases of sepsis, severe leptospirosis, preeclampsia, and pancreatitis [32, 68–71]. Further, we showed oleic acid-induced lung injury in mice via pulmonary and extrapulmonary routes that are similar to ARDS [36, 38]. Additionally, ARDS patients presented elevated oleic acid levels in the blood and lung [68], and this fact supports the idea that oleic acid has a critical role in ARDS pathogenesis (Figure 1).

Even though none of animal models fully mimics findings in the human disease, studies using animal ARDS models endure a vital biological tool to study the pathophysiology of and to test novel therapeutic interventions. It is easily reproducible and reliable and, therefore, a powerful model to study lung injury mechanisms and putative candidates in the ARDS treatment.

9. Conclusion

Oleic acid-induced lung injury is a relevant model to study ARDS because this fatty acid acts directly on the lung cells or lung endothelium and triggers activation of different innate immune receptors. It leads to cell activation, inflammatory mediator production, and cell death (Table 3), thus closely mimicking human ARDS. Further, NKA inhibition by oleic acid likely plays a critical role in lung injury during conditions of high oleic acid plasma levels, such as sepsis, leptospirosis, pancreatitis, and preeclampsia. Several key features of ARDS could be explored in the animal model of oleic acid-induced lung injury. Oleic acid concentration in plasma and/or BALF of patients is a critical predictor of ARDS development or outcome and, therefore, it could be used as a biological marker of disease severity. Therefore, the oleic acid model is likely suitable for studying the pathophysiology of ARDS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Mauro Velho Castro-Faria and Hugo Caire Castro-Faria-Neto contribute equally to this work.

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Review Article

Sepsis and ARDS: The Dark Side of Histones

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Despite advances in management over the last several decades, sepsis and acute respiratory distress syndrome (ARDS) still remain major clinical challenges and the leading causes of death for patients in intensive care units (ICUs) due to insufficient understanding of the pathophysiological mechanisms of these diseases. However, recent studies have shown that histones, also known as chromatin-basic structure proteins, could be released into the extracellular space during severe stress and physical challenges to the body (e.g., sepsis and ARDS). Due to their cytotoxic and proinflammatory effects, extracellular histones can lead to excessive and overwhelming cell damage and death, thus contributing to the pathogenesis of both sepsis and ARDS. In addition, antihistone-based treatments (e.g., neutralizing antibodies, activated protein C, and heparin) have shown protective effects and have significantly improved the outcomes of mice suffering from sepsis and ARDS. Here, we review researches related to the pathological role of histone in context of sepsis and ARDS and evaluate the potential value of histones as biomarkers and therapeutic targets of these diseases.

1. Introduction

Over the last several decades, severe sepsis and acute respiratory distress syndrome (ARDS) have been the most common causes of mortality in critically ill patients [1–3]. During these years, a growing number of advanced interventions and strategies have been applied to critically ill patients. Pharmacological interventions, including antithrombin III [4], tifacogin [5], vasoactive drugs [6, 7], and activated protein C [8], have been proven to be helpful. Moreover, the strategies of mechanical ventilation are of vital importance. With an increasing use of noninvasive positive-pressure ventilation, a reduction in tidal volume, and an increase in applied positive end-expiratory pressure [9], the mortality of critically ill patients with sepsis and ARDS has gradually decreased over the last decade [9, 10]. However, the mortality rates still remain unacceptably high, with a 20 to 30% mortality rate from sepsis [11] and a mortality rate greater than 40% from ARDS [12].

Despite advanced developments in life support management (e.g., ventilators, dialysis, and extracorporeal membrane oxygenation), these interventions are not specific for blocking or targeting the pathogenic processes of these diseases. Therefore, a comprehensive treatment for critical illness should include not only alleviating the pain but also targeting the underlying pathological mechanism. However, the underlying mechanisms of ARDS and sepsis remain largely unknown. Sepsis and ARDS result from complex events such as infections, trauma, burning, and acid aspiration [13], which trigger innate and adaptive immune responses. The complexity of these processes involves complement system activation, neutrophil infiltration, vascular endothelial system damage, coagulation cascades promotion, and barrier dysfunction [14, 15]. Therefore, for a better understanding of the pathophysiological process of sepsis and ARDS, additional molecular mechanisms need to be explored.

It appears to be widely accepted that investigating the targets that are abnormally expressed in critically ill patients and in animal models holds promise for identifying new underlying molecular mechanisms. Recently, it has been reported that histones, as basic and important structural

elements in nuclear chromatin and the regulation of gene transcription, can be released passively into the extracellular space when cells undergo severe injury, giving rise to immunostimulatory and cytotoxic effects on both sepsis [16, 17] and ARDS [18, 19].

Before they are released into the extracellular space, histones are the major proteins of chromosomes found in eukaryotic cell nuclei and are highly conserved across species. There are five families of histones known to date: H2A, H2B, H3, and H4, which are known as "core histones," and histone H1 and its homolog H5, which are known as the linker histones [20–22]. Histones are the basic structural elements in the nucleosome, which contains one H3/H4 tetramer and two H2A/H2B dimers, while H1 binds to nonnucleosomal DNA and facilitates numerous nucleosomes to form higher-order chromatin structures [20, 23]. Even though histones are extremely inert in the nucleus, they lead to significant pathogenic effects outside of the cells.

Mounting evidence from clinical and experimental data indicates that extracellular histones could act as new members of damage-associated molecular pattern molecules (DAMPs) [24–26]. The results from both patients and animal models have suggested that circulating histones play a crucial role in sepsis and ARDS and could serve as novel biomarkers as well as promising therapeutic targets [27, 28]. Therefore, a deeper understanding of the functions of extracellular histones may yield pivotal insights into the pathogenesis of sepsis and ARDS. In this review, we will focus on the pathogenic effects and clinical relevance of extracellular histones and hope to help set the stage for future studies.

2. The Source of Extracellular Histones

The source of extracellular histones is complicated. Histones are reported to be released from dying cells [29, 30]. During necrosis, accompanied by disruption of the cell plasma membrane, intracellular components are released into the extracellular space, and some (e.g., HMGB1, DNA, and histones) have the ability to activate innate immunity and cause more injury. Although apoptotic cells are in silent death without membrane disintegration [31], they are also thought to release histones by leaking from membrane blebs [32] and nucleosomes [33], which are produced by actin-myosin contractions during apoptosis.

In addition, the release of histones is also considered to be associated with neutrophil extracellular traps (NETs) [34]. NETs are formed by dying neutrophils that release DNA, histones, and granular proteins, such as neutrophil elastase and myeloperoxidase. In this way, the released histones play a predominant role in further inducing epithelial and endothelial cell death [35]. Therefore, extracellular histones can also be released by forming NETs. Another possible source of histones is large numbers of apoptotic and necrotic cells overwhelming the clearance ability of mononuclear phagocytes, thereby allowing histones to enter the circulatory system [36].

3. The Receptors of Extracellular Histones

Toll-like receptors, including toll-like receptors 2, 4, 9 (TLR2, TLR4, and TLR9), have been shown to be receptors of extracellular histones [25, 30, 37–39]. For example, histones promote plasma thrombin generation via TLR2 and TLR4 activation [39]. In addition, in the context of acute kidney injury, histones can induce leukocyte accumulation, renal inflammation, and microvascular leakage in TLR2/TLR4 dependent mechanism [30]. Moreover, extracellular histones are mediators of death in inflammatory injury and in chemical-induced cellular injury through TLR2 and TLR4 signaling [38]. Furthermore, endogenous histones mediate sterile inflammatory liver injury via TLR9 in mice [37]. However, Abrams and his colleagues [18] suggested that blockading TLR4 and TLR2 in trauma-associated lung injury models showed no protective effects, indicating that the activation of TLR2 and TLR4 may not be major pathway responses for histone toxicity. Collectively, in different disease models, extracellular histones may activate different toll-like receptors, including TLR2, TLR4, and TLR9, to mediate various pathogenic effects.

However, activated protein C (APC) and specific antibodies to histones can significantly reduce cytotoxicity and the mortality of septic mice by hydrolyzing or neutralizing histones, respectively [16, 18]. In addition, the protective effects of blockading TLR4 and TLR2 remain controversial. A report by Xu et al. [38] showed that TLR4 knock-out mice were protected from the fatal effects of histone infusion, and Ekaney et al. [17] demonstrated that blockading TLR4 decreased cellular cytotoxicity in endothelial cells. By contrast, Abrams and his colleagues [18] suggested that blockading TLR4 and TLR2 could not block a calcium influx when endothelial cells were treated with histones. These results indicate that TLR2 and TLR4 are receptors of histones. Blockading TLR2 and TLR4 may be protective; however, the exact mechanisms may differ in different disease models, and further investigation is needed.

4. Pathologic Roles of Extracellular Histones in Sepsis

Sepsis is a systemic inflammatory response to infection [40]. During the past two decades, it has remained an important clinical challenge in the intensive care unit (ICU) and one of the leading causes of death [41] due to an incomplete understanding of its pathophysiological mechanisms. Numerous studies in the field of sepsis have identified host response, innate immunity, coagulation abnormalities, and the balance between proinflammation and anti-inflammation as essential contributors to sepsis. Recently, Xu et al. [16] and Ekaney et al. [17] demonstrated that extracellular histones were major mediators in endotoxemia and septic shock through cytotoxicity, excessive inflammation, and coagulation dysfunction.

4.1. Cytotoxic Effects. High levels of extracellular histones are cytotoxic to both epithelial and endothelial cells [16, 18, 19, 35, 42]. Xu et al. [16] treated endothelial cells, specifically EA.hy926, with a mixture of purified mixed histones and five

individual histones. They found that a mixture of histones was cytotoxic to these cells and the toxic effects were mainly due to histones H3 and H4. In addition, Abrams and his colleagues [18] demonstrated that sera from patients were toxic to cultured endothelial cells once histone levels exceeded 50 μg/mL. Sera from sepsis patients directly induced histone-specific cardiomyocyte death, which further contributed to the development of cardiac injury, arrhythmias, and left ventricular dysfunction [43]. Interestingly, extracellular H1, but not H2A/H2B, H3, and H4, is neurotoxic and induces dramatic neuronal death [44].

However, the mechanism of the toxic effect of extracellular histones is not completely clear. It has been reported that positively charged histones could bind to negatively charged phospholipids in the plasma membrane [18, 45], leading to increased transmembrane conductance [46, 47], membrane disruption [18] and, finally, calcium influx [18, 48, 49]. Moreover, lymphocyte apoptosis induced by histones during sepsis is dependent on p38 phosphorylation and mitochondrial permeability transition [50]. Further studies have found that sera from survivors of septic shock were able to specifically induce dendritic cell (DC) apoptosis in a caspase-dependent pathway, and sera from nonsurvivors were able to induce DC-regulated necrosis, which could be abrogated by antihistone therapy [51].

4.2. Triggering and Promoting Inflammation in Sepsis. The innate immune system plays a crucial role in the pathophysiology of sepsis, which induces overwhelming systemic inflammation by releasing various inflammatory mediators in response to invading pathogens [14, 52]. In addition, histones could serve damage-associated molecular pattern molecules [53] involved in the aggravation of systemic inflammation. Recent studies have demonstrated that the release of histones contributes to the considerable production of sepsis-associated cytokines, such as TNF-α, IL-6, and IL-8, as well as IL-1β, and leads to cytokine storm [17, 54]. There are several reasons for this, detailed below.

First, histones could interact with TLR2 and TLR4 as ligand receptors and directly activate myeloid differentiation primary response gene88 (MyD88) to initiate inflammation [30, 38]. However, there may be some differences between histones and TLR9 interaction. TLR9 is an intracellular molecule that functions as a receptor of DNA [55, 56] and, therefore, histones bind to DNA and then enter the intracellular space to enhance the DNA-activated TLR9 signaling cascade [37].

Second, histones can activate monocyte-derived dendritic cells via the NLRP3 inflammasome to induce the production of IL-1β [45, 57, 58]. Lipopolysaccharide (LPS) pretreatment, followed by the addition of histones, showed significantly amplified production of IL-1β from the wild-type macrophages but not from NLRP3-defected macrophages, indicating that histones activated the NLRP3 inflammasome in macrophages to induce the release of IL-1β [59]. Activating the NLRP3 inflammasome with histones could promote the recruitment of neutrophils and the additional release of histones into the extracellular space, which establishes a vicious cycle that enhances inflammation [59, 60].

Third, histone-induced inflammation can be amplified by DNA and polyphosphate [37, 61]. Extracellular histones can enhance TLR9-mediated inflammation by interacting with DNA [37]. Moreover, polyphosphate amplifies H4-mediated inflammation in human umbilical vein endothelial cells specifically through interaction with the receptor for advanced glycation end products (RAGE) and P2Y1 [61].

Last, the charge itself may have proinflammatory effects [45]. Histones with highly positive charges are responsible for cytotoxicity and barrier dysfunction by charge-charge interaction [18]. In this regard, the geometry, topology, or density of the charge may determine the immune activity [45], but further investigations are needed.

4.3. Coagulation and Thrombosis in Sepsis. Sepsis is almost inevitably associated with the activation of blood coagulation (hypercoagulability) and systemic clotting with massive thrombin and fibrin formation, eventually resulting in the consumption of platelets and disseminated intravascular coagulation (DIC) [36, 62, 63]. Recent reports have suggested that extracellular histones triggered platelet aggregation and clotting both in vivo and in vitro [39, 64–67]. Therefore, by the consumption of platelets, histone-treated mice showed thrombocytopenia, prolonged prothrombin time, decreased fibrinogen, fibrin deposition in the microvasculature, and DIC bleeding [65, 68].

A growing body of evidence reveals that the impact of histones on the above responses is not only related to charge [69] but also mediated through the activation of TLR2 and TLR4 signaling pathways (e.g., ERK, Akt, p38, and NF-κB), the induction of calcium influx, and fibrinogen recruitment [39, 67]. Moreover, histones can increase plasma thrombin generation by reducing protein C activation [70]. Blockading platelets TLR2 and TLR4 with antibodies decrease both the activated platelets and the thrombin generation [39]. Histone-related platelet activation can be prevented in vitro and in vivo by pretreatment with low-dose heparin, which directly antagonizes histones rather than causing anticoagulation [71]. Other therapies also proven to be effective include APC [16], albumin [66], globular C1q receptor (P33) [72], recombinant thrombomodulin (rTM) [68], chondroitin sulfate (CS), and high molecular weight hyaluronan (HMW-HA) associated with the interalpha inhibitor protein (IAIP) [73].

Apparently, a number of the blockers mentioned above, including heparin, albumin, IAIP and HMW-HA, carry negative charges. They can significantly reduce cytotoxicity and platelets activation by neutralizing positive charges and binding with histones. Therefore, we find it reasonable to speculate that negatively charged molecules may naturally have a potent antihistone capacity, which is promising for the development of pharmaceutical drugs to cure histone-related diseases (Table 1).

5. Pathologic Roles of Extracellular Histones in ARDS

Despite advances in management over the last several decades, acute respiratory distress syndrome (ARDS)

Table 1: Sepsis-associated organ dysfunction induced by extracellular histones.

Organ dysfunction	Mechanism	Reference
Lung injury	Cytotoxicity, NLRP3 inflammasome	[42, 59, 74]
Cardiac injury	Cytotoxicity	[43, 60, 74]
Liver injury	Proinflammation	[74, 75]
Kidney injury	Proinflammation, cytotoxicity	[30, 74]
Spleen injury	Cytotoxicity	[74, 76]
Coagulation	Platelets activation, thrombosis	[39, 63, 67]

remains an important clinical challenge due to the incomplete understanding of its pathophysiological mechanisms. Recently, a growing body of evidence suggests that extracellular histones contribute to the pathogenesis of ARDS. Histones appear in the bronchoalveolar lavage fluids (BALF) and plasma of patients who developed ARDS after trauma and acid aspiration [18, 19]. By carefully studying these findings, it was determined that the lungs are the most susceptible organ to high levels of circulating histones [18].

5.1. Induction of ARDS and Requirements for C5aR/C5L2. Complement component C5a displays the highest inflammatory potency for inducing inflammation [77], which is believed to be involved in the induction of ARDS [42, 78, 79]. As expected, in experimental mice models following airway deposition of LPS or C5a, extracellular histones appear in both the BALF and plasma [42]. However, the presence of extracellular histones is significantly reduced when the mice are knocked out either C5a receptors (C5aR and C5L2) or by the depletion of neutrophils or macrophages [42]. Interestingly, once the histones are present in the extracellular space, cytokine production, epithelial cell damage, barrier dysfunction, and the coagulation cascade activation, which are induced by histones, are independent of C5a receptors [42]. Together, these data indicate that the extracellular histones' appearance requires C5aR/C5L2. However, neutrophil accumulation sometimes occurs with infection without complement activation. It is reported that the absence of C3 or C5 affected neither the accumulation of neutrophils in the lungs nor their appearance in the alveolar space in airway deposition of LPS-induced ALI model [80, 81].

5.2. Critical Role for the NLRP3 Inflammasome during ALI. The NLRP3 inflammasome is a multiprotein complex that activates caspase-1, giving rise to the maturation of the proinflammatory cytokines, including IL-1β and IL-18, and the induction of pyroptosis [82, 83]. A recent study has suggested an essential role of NLRP3 inflammasome in the development of experimental ALI, as NLRP3 or caspase-1 knock-out mice showed significantly reduced amounts of neutrophil infiltration and albumin leakage in different models of ALI [59]. In addition, extracellular histones can directly activate the NLRP3 inflammasome via the generation of reactive oxygen species as well as the extrusion of K^+ and the elevation of intracellular Ca^{2+} concentration [57–59]. Moreover, NLRP3 and caspase-1 are also required for the presence of extracellular histones during ALI [59], indicating positive feedback and a potential mechanism for inflammatory propagation. Such findings reveal a dynamic interaction between NLRP3 inflammasome and extracellular histones that contributes to ALI.

5.3. Barrier Dysfunction and Permeability Changes Induced by Extracellular Histones. The pathology of ARDS is characterized by an acute inflammatory response linked to the overwhelming recruitment and accumulation of neutrophils, fibrin deposits, alveolar hemorrhage, and pulmonary edema fluid [13, 84, 85]. Recent studies have shown that extracellular histones are responsible for pulmonary edema, which is characterized by increased endothelial and epithelial permeability [18, 42, 86]. In vivo, airway administration of calf thymus histones led to a dose-dependent disruption of the alveolar permeability barrier during ALI, with observations of alveolar albumin leakage and a histological examination revealing obvious lung edema [18, 42]. In vitro, compared with controls, transwells plated with endothelial cells by pretreatment with histones showed that FITC-labeled albumin was significantly elevated in the lower wells, which indicates a histone-induced endothelial permeability increase [18, 86]. Furthermore, recombinant parasite histones also induced endothelial permeability via a charge-dependent mechanism that led to downregulation of the junction protein [86]. Taken together, these data suggest that extracellular histones play a crucial role in barrier dysfunction during ALI. However, in addition to the charge-dependent mechanism, TLR2, TLR4, and TLR9, as the receptors of histones [30, 37, 38], may also give rise to permeability changes, which should be investigated further (Figure 1).

6. Clinical Relevance of Plasma and BALF Histones

High concentrations of plasma histones have been detected in patients with sepsis [16, 17] and ARDS [18, 87] and, possibly, correlate with the severity or poor prognosis of these diseases [19, 88]. As observed by Ekaney et al. [17], in septic patients, large amounts of histones are significantly linked to lower endogenous APC levels, a decrease in platelet count, and the need for renal replacement therapy (RRT). Extracellular histones have also been found to predict ICU 28-day mortality in patients with sepsis, and the area under curve (AUC) is 0.744 ($p = 0.003$) with a histone cutoff value of 75 μg/mL (sensitivity 60% and specificity 86.1%) [43]. Moreover, high levels of circulating histones in septic patients are associated with a higher prevalence of new-onset left ventricular dysfunction and arrhythmias (AUC = 0.865, $p = 0.001$ and AUC = 0.813, $p = 0.001$, resp.) [43]. Similarly, in patients with trauma, elevated histone levels are associated with acute lung injury, more days of mechanical ventilation,

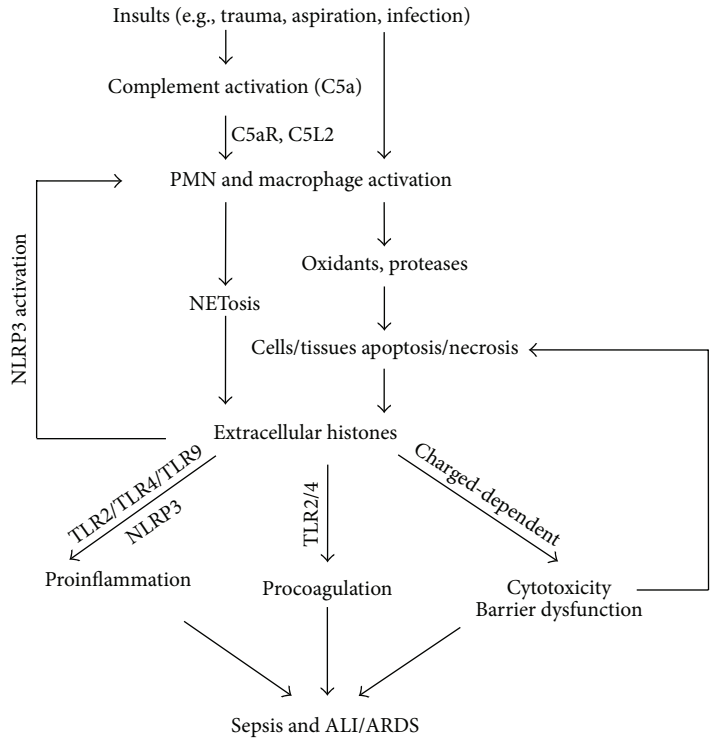

Figure 1: Proposed mechanisms of extracellular histones in the development of sepsis and ALI/ARDS. In response to various physical challenges (e.g., trauma, infection), polymorphonuclear neutrophils (PMN) and macrophages are recruited and activated through complement interaction (C5a and C5a receptors), which is often needed for extracellular histones presented in ALI/ARDS models. However, the accumulation of PMNs sometimes occurs with infection without complement activation. Under these conditions, histones derived from NETosis and dying nonleukocytic cells could be released. Once the histones are present in the extracellular space, they can directly bind to and damage phospholipids in cell membranes in a charged-dependent mechanism, leading to increased membrane permeability and death. They can also act on TLR2, TLR4, and TLR9 and activate the NLRP3 inflammasome to amplify inflammatory responses by the growing release of cytokines and other mediators. Moreover, circulating histones may also enhance coagulation disorders by acting on TLR2 and TLR4. On the other hand, extracellular histones perpetuate detrimental cell/tissue injury and could in turn induce the formation of NETs by activating the NLRP3 inflammasome, which together lead to more histones being released and greater severity of sepsis and ALI/ARDS.

higher incidences of organ failure, and even higher mortality. An increasing histone level from arrival to 6 h after admission was a multivariate predictor of mortality (hazard ratio 1.005, $p = 0.013$) [88]. In addition, extracellular histones indicate higher mortality in patients with gastric aspiration-induced ARDS [19]. However, extracellular histones are only detectable in 50% of ARDS patients' bronchoalveolar lavage fluid (BALF) from 0 to 10 days after diagnosis. Lower rates of histones are present in BALF samples collected >10 days after diagnosis [42], indicating that histones may only be present in early samples. This might result from treatment with heparin in ICU patients, especially when they receive RRT [17]. Heparin is a highly negatively charged molecule and may bind to positively charged histones to reduce both their cytotoxicity and the number of extracellular histones [89, 90]. Collectively, extracellular histones are significantly elevated in critical diseases, such as sepsis and ARDS, and can reflect severity and mortality, potentially making them a useful and promising biomarker and a therapeutic target.

7. Extracellular Histones as Therapeutic Targets

Despite considerable studies into the molecular mechanisms and treatment trials for sepsis and ARDS, the unequivocal and solid curative effect remains limited. However, an increasing body of evidence reveals that histone-related sepsis and ARDS can be inhibited by histone-neutralizing antibodies [27, 30, 38, 91]. For instance, LG2-1 recognizes a peptide from histone H3, LG2-2 reacts with the aminoterminus of H2B, and BWA3 binds to H2A and H4 [92]. More recent studies from Kusano et al. demonstrated that a novel antihistone H1 monoclonal antibody, the SSV monoclonal antibody (SSV mAb), could not merely bind to histone H1 but also exhibited cross-reactivity against histones H3 and H4 [93]. In addition, agonistic activity on TLR2, TLR4, TLR9, and the NLRP3 inflammasome may also provide a potential way to target histones for therapy [30, 37, 38, 57–59]. Moreover, the appearance of extracellular histones requires C5aR/C5L2,

and, thus, neutralizing C5a or blockading C5aR/C5L2 may be a potent target that limits the release of histones [42, 76]. As a result of amplifying histone-mediated inflammation through interaction with RAGE and P2Y1 by polyphosphate, targeting polyphosphate, RAGE, and P2Y1 might also have favorable prospects [61]. Moreover, targeting positive charges of histones may be crucial and beneficial because a number of studies have revealed that negatively charged molecules, including heparin [19, 89, 90], albumin [66], C-reactive protein (CRP) [94], endothelial surface protein/gC1q receptor (P33) [72], CS associated IAIP, and HMW-HA [73, 95], could directly bind with histones and abrogate the histone-related pathology. It appears that negatively charged molecules may naturally have a potent antihistone capacity, which is a promising and positive target that needs further investigation. Furthermore, pentraxin 3 (PTX3) also exerts protective effects on sepsis, both in vivo and in vitro, due to its coaggregation with histones [96]. Recombinant thrombomodulin (rTM) could bind to extracellular histones, inhibiting histone-induced platelet aggregation and neutralizing the prothrombotic action of histones [68]. Finally, although FDA-cleared recombinant APC has been withdrawn from the market because of a lack of efficacy in reducing the mortality of sepsis by randomized controlled trials [97], the exact role of APC in hydrolysis and the inactivation of histones has been identified and shows great benefits in a number of experimental studies [16, 30, 86, 98]. Therefore, the appropriate and safe use of APC may still be promising in the early stages of sepsis and ARDS. However, more animal models and clinical randomized controlled trials are needed (Table 2).

Table 2: Current evidence of targeting extracellular histones for therapy.

Antibody or molecule	Mechanism	References
SSV mAb	Bind to H1; cross-reactivity against H3, H4	[93]
LG2-1	Neutralize H3	[18, 35, 86, 92]
LG2-2	Neutralize H2B	[18, 35, 86, 92]
BWA3	Neutralize H2A and H4	[18, 35, 86, 92]
Anti-TLR2/TLR4/TLR9	Blockade of TLR2/TLR4/TLR9 receptors	[30, 37, 38]
Heparin	Negative charge	[89, 90]
Albumin	Negative charge	[66]
CRP	Negative charge	[94, 99]
SAP	Negative charge	[99]
P33	Negative charge	[72]
IAIP	Negative charge	[73]
HMW-HA	Negative charge	[73, 95]
PTX3	Coaggregation with histones	[96]
rTM	Inhibit histone-induced platelet aggregation	[68]
APC	Degrade histones	[16, 30, 86, 98]

TLR = toll-like receptor, CRP = C-reactive protein, SAP = serum amyloid P component, P33 = endothelial surface protein/gC1q receptor, IAIP = interalpha inhibitor protein, HMW-HA = high molecular weight hyaluronan, PTX3 = pentraxin 3, rTM = recombinant thrombomodulin, and APC = active protein C.

8. Conclusions and Perspectives

In summary, histones, as the main structure elements, have recently been identified to be present in the extracellular space and to be involved in multiple cellular processes, including cytotoxicity, proinflammation, procoagulation, and barrier dysfunction. Therefore, extracellular histones can help with diagnosis, predict prognosis, and reflect the severity of critical illnesses, including sepsis, ARDS, and septic-ARDS. Antihistone-based therapeutic strategies are thought to be useful and promising. However, there are still many unanswered questions regarding how and when histone-blocking agents should be used and the additive effects of combining different histone-targeted agents. Therefore, the appropriate and safe use of different antihistone-based agents still needs further investigation. Moreover, a better understanding of the substructure, modification modes, and regulation and function of histones in the extracellular space is still needed.

Conflict of Interests

The authors declare that they have no competing interests.

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Research Article

Airway β-Defensin-1 Protein Is Elevated in COPD and Severe Asthma

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Background. Innate immune antimicrobial peptides, including β-defensin-1, promote the chemotaxis and activation of several immune cells. The role of β-defensin-1 in asthma and chronic obstructive pulmonary disease (COPD) remains unclear. *Methods*. Induced sputum was collected from healthy controls and individuals with asthma or COPD. β-defensin-1 protein in sputum supernatant was quantified by ELISA. Biomarker potential was examined using receiver operating characteristic curves. β-defensin-1 release from primary bronchial epithelial cells (pBECs) was investigated in culture with and without cigarette smoke extract (CSE). *Results*. Airway β-defensin-1 protein was elevated in COPD participants compared to asthma participants and healthy controls. Inflammatory phenotype had no effect on β-defensin-1 levels in asthma or COPD. β-defensin-1 protein was significantly higher in severe asthma compared to controlled and uncontrolled asthma. β-defensin-1 protein could predict the presence of COPD from both healthy controls and asthma patients. Exposure of pBECs to CSE decreased β-defensin-1 production in healthy controls; however in pBECs from COPD participants the level of β-defensin-1 remanied unchanged. *Conclusions*. Elevated β-defensin-1 protein is a feature of COPD and severe asthma regardless of inflammatory phenotype. β-defensin-1 production is dysregulated in the epithelium of patients with COPD and may be an effective biomarker and potential therapeutic target.

1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory airway diseases, characterised by airflow limitation obstruction that is reversible in asthma yet, under current therapeutics, progressive and not completely reversible in COPD. The global burden imposed by these diseases is considerable [1]. Asthma and COPD are recognized for their heterogeneous nature, particularly in regard to the type of inflammation present [2]. Four major phenotypes (eosinophilic, neutrophilic, paucigranulocytic, and mixed granulocytic) have been described and characterised by the proportions of eosinophils and neutrophils [2, 3]. These inflammatory phenotypes are associated with differences in disease severity and response to corticosteroids [4, 5], yet their underlying biology remains poorly understood [6, 7].

The migration and activity of eosinophils and neutrophils are influenced by a range of host factors, including a class of antimicrobial peptides also known as alarmins. These antimicrobial peptides are small (<100 amino acids) proteins, which play an important role in influencing and modulating the immune response through the receptor-mediated chemotaxis and activation of a range of innate and adaptive immune cells [8]. Defensins and cathelicidin constitute key alarmin families in humans [8]. The α- and β-defensins constitute the two major classes of human defensins, classified based on the differential organisation of six cysteine motifs [9]. β-defensin-1 is constitutively expressed by the epithelial cells of the respiratory tract and is both broadly antimicrobial and able to influence the immune response [10].

β-defensin-1 polymorphisms have been associated with both asthma [11, 12] and COPD [13, 14]. Increased gene

expression of β-defensin-1 has been observed in bronchial epithelium and BAL fluid cells in COPD and was negatively associated with lung function and airflow limitation [15]. β-defensin-1 protein, however, has yet to be examined in either asthma or COPD. This study characterises the protein levels of β-defensin-1 in induced sputum samples in both asthma and COPD compared with healthy controls. We investigate the relationship of β-defensin-1 to inflammatory phenotypes and disease severity. To understand the potential source of aberrant β-defensin-1 expression, we also investigate its production in primary bronchial epithelial cells exposed to cigarette smoke extract in culture. We hypothesised that protein expression would be greater in asthma and COPD, associated with disease severity and inflammatory phenotype, and altered in epithelial cells after cigarette smoke exposure.

2. Methods

2.1. Study Design and Sputum Analysis. Adults with COPD and asthma were recruited from the John Hunter Hospital Ambulatory Care Clinic, NSW, Australia. Participants with COPD ($n = 43$ and $n = 10$ for the primary bronchial epithelial cell (pBEC) study) had a diagnosis of COPD and postbronchodilator $FEV_1/FVC < 70\%$. Participants with asthma ($n = 94$) were diagnosed according to American Thoracic Society guidelines based upon current (past 12 months) episodic respiratory symptoms, doctor's diagnosis, and demonstrated evidence of airway hyperresponsiveness to hypertonic saline. Healthy controls ($n = 28$ and $n = 4$ for the pBEC study) were nonsmokers and had $FEV_1 > 80\%$ predicted and were recruited by advertisement. Exclusion criteria included a respiratory tract infection, exacerbation of respiratory disease or change in maintenance therapy in the past month, and current smoking (except for the pBEC study where current smoking in the COPD group was not exclusion criteria). All participants gave written informed consent and the Hunter New England Area Health Service and The University of Newcastle Research Ethics Committees approved this study.

Spirometry (KoKo PD Instrumentation, Louisville, Colorado, USA) and sputum induction with hypertonic saline (4.5%) were performed in participants with $FEV_1 > 1.3$ L and with 0.9% saline in participants whose FEV_1 was below this level. The protocol specified a fixed sputum induction time of 15.5 minutes. For inflammatory cell counts, selected sputum was dispersed using dithiothreitol (DTT), suspension was filtered, and a total cell count and cell viability count were performed. Cytospins were prepared and stained (May-Grunwald Giemsa) and a differential cell count was obtained from 400 nonsquamous cells.

2.2. Measurement of β-Defensin-1 Protein. The concentration of β-defensin-1 was determined by ELISA (100-240-BD1, Alpha Diagnostic International, San Antonio, Texas, USA) as per manufacturer's instructions. The standard curve ranged from 50 to 800 pg/mL. The measurement of β-defensin-1 protein in sputum supernatant was validated by determining the following: the inhibitory effect of DTT, matrix effects of sample dilution, and the recovery of spiked protein. DTT had an insignificant effect on the standard curve. Matrix effects were minimised when samples were diluted 1/10. On average, 104% of spiked β-defensin-1 protein ($n = 6$) was recovered.

2.3. Disease Classifications. The granulocyte cut-offs to determine inflammatory phenotype used were ≥3% for sputum eosinophils and ≥61% for sputum neutrophils [2]. Severe asthma subjects had uncontrolled asthma (measured by Juniper Asthma Control Questionnaire (ACQ), score ≥ 1) and/or poor lung function (FEV_1% predicted ≤80 and $FEV_1/FVC\% \leq 70$) despite prescription of high-dose inhaled corticosteroids (ICS) (>1000 μg beclomethasone equivalents per day) in combination with long acting β-agonists [16]. If participants had poor lung function or poor symptom control but did not meet the treatment requirements for severe asthma, they were classified as uncontrolled asthma. Controlled asthma was defined as normal lung function (FEV_1% predicted > 80 and/or $FEV_1/FVC\% > 70$) and controlled symptoms (ACQ score < 1). COPD severity was defined according to the global obstructive lung disease (GOLD) initiative stage [17].

2.4. Primary Bronchial Epithelial Cell (pBEC) Culture. Cigarette smoke extract (CSE) was prepared by bubbling smoke from one filterless Kentucky research cigarette, 3R4F containing 9.5 mg tar and 0.8 mg nicotine, through 10 mL of cell culture medium Bronchial Epithelial Basal Medium (BEBM, Lonza) at a speed of 5 minutes per cigarette and used in the following cell culture experiments immediately. Human pBECs were obtained by endobronchial brushing during fibre-optic bronchoscopy and cultured as described previously [18]. pBECs were maintained in Bronchial Epithelial cell Growth Medium (Lonza). Cells were seeded onto placental collagen (Sigma) coated 24-well plates (Nunclon) and used at passage 2 once they reached 80% confluency. After exposure to 1% CSE the pBECs were maintained in BEBM (Lonza) containing 1x insulin, transferrin, and sodium selenite liquid media supplement (Sigma). We have previously determined that this concentration of CSE via dose response curves causes minimal toxicity to the cells whilst still inducing an immune response. All cells were grown at 37°C with 5% CO_2 in air. Cell culture supernatant was collected at 24 hrs for assessment of β-defensin-1 protein.

2.5. Statistical Analysis. Clinical and cell count data were analysed using Stata/IC 11.1 (Stata Corporation, College Station, Texas, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., California, USA) and reported as mean (SD) for normally distributed data and median (Q1 and Q3) for nonparametric data. For normally distributed data, t-test or ANOVA test with a Bonferroni post hoc test to adjust for multiple comparisons was applied. For nonparametric data, a Mann-Whitney test or Kruskal-Wallis test with Dunn's post hoc test to adjust for multiple comparisons was applied. Statistical comparisons for categorical data were made using a χ^2 test. Associations between variables were assessed using

Table 1: Clinical characteristics and sputum cell counts in patients with inflammatory airway diseases compared to healthy controls.

	Healthy controls	Asthma	COPD	p value
N	28	94	43	
Age (years), mean (SD)	46 (19)	57 (13)	70 (8)†‡	<0.001
Gender, M/F	12/16	38/56	23/20	0.356
Atopy, n (%)	13 (46)	62 (66)	23 (53)	0.119
FEV_1, % predicted, mean (SD)	107 (13)	79 (21)†	55 (16)†‡	<0.001
FEV_1/FVC, %, mean (SD)	81 (7)	69 (10)†	54 (11)†‡	<0.001
BMI, kg/m^2, mean (SD)	26.1 (4)	30.6 (7)†	29.3 (7)	0.008
Exhaled nitric oxide (pbb), median (Q1, Q3)	18.6 (14.6, 22.3)	21.6 (14.8, 21.6)	18.1 (14.5, 23.8)	0.127
Smoking, ex/never	9/19	38/56	33/10†‡	<0.001
Pack years, median (Q1, Q3)	17 (3, 45)	7 (3, 20)§	32 (20, 54)	<0.001
Inhaled corticosteroid (ICS) use, n (%)	NA	77 (82)	36 (84)	0.798
ICS dose (μg daily BDP equivalent) median (Q1, Q3)	NA	1000 (500, 2000)	2000 (1000, 2000)	0.012
Long acting β-agonist use, n (%)	NA	76 (81)	34 (79)	0.808
Long acting muscarinic receptor antagonist (LAMA) use, n (%)	NA	13 (14)	24 (56)	<0.001
Total cell count $\times 10^6$/mL, median (Q1, Q3)	2.5 (1.3, 4.5)‡§	3.4 (2.1, 7.1)	4.7 (2.9, 9.9)	0.007
Viability, median (Q1, Q3)	75.0 (64.3, 88.1)	77.6 (68.1, 90.9)	81.5 (75.8, 93.1)	0.142
Neutrophils, %, median (Q1, Q3)	29.0 (10.1, 52.3)	43.8 (24.8, 63.0)†	73.1 (46.0, 85.5)†‡	<0.001
Neutrophils $\times 10^4$/mL, median (Q1, Q3)	55.6 (24.5, 134.4)	128.3 (53.5, 324.0)†	339.1 (136.6, 705.9)†‡	<0.001
Eosinophils, %, median (Q1, Q3)	0.3 (0.0, 0.5)	1.0 (0.3, 4.3)†	0.8 (0.4, 2.3)†	0.001
Eosinophils $\times 10^4$/mL, median (Q1, Q3)	0.4 (0.0, 1.9)	5.6 (0.5, 29.8)†	6.3 (1.5, 14.4)†	<0.001
Macrophages, %, median (Q1, Q3)	66.1 (42.2, 84.0)	43.3 (24.3, 58.5)†	18.9 (9.0, 49.3)†‡	<0.001
Macrophages $\times 10^4$/mL, median (Q1, Q3)	150.1 (79.4, 257.5)	137.9 (80.6, 218.7)	111.8 (54.0, 169.7)	0.187
Lymphocytes, %, median (Q1, Q3)	0.9 (0.3, 2.0)	0.5 (0.3, 1.3)	0.3 (0.0, 0.5)†‡	0.002
Lymphocytes $\times 10^4$/mL, median (Q1, Q3)	1.9 (0.4, 4.7)	1.9 (0.2, 5.0)	0.5 (0.0, 1.7)†‡	0.009
Columnar epithelial cells, %, median (Q1, Q3)	1.9 (0.5, 7.5)	1.6 (0.3, 4.0)	1.0 (0.3, 2.0)	0.104
Columnar epithelial cells $\times 10^4$/mL, median (Q1, Q3)	3.1 (1.5, 11.7)	3.7 (1.6, 10.9)	3.4 (1.3, 10.1)	0.959

$^{\dagger}p < 0.05$ versus healthy, $^{\ddagger}p < 0.05$ versus asthma, and $^{\S}p < 0.05$ versus COPD.
BMI: body mass index; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; NA: not applicable.

Spearman correlation coefficients. Receiver operating characteristic (ROC) curves were generated and the area under the curve (AUC) was calculated to assess the relationship between the presence of COPD and β-defensin-1 levels.

3. Results

3.1. Clinical Features and Inflammatory Cells in Asthma Participants, COPD Patients, and Healthy Controls. Clinical details and inflammatory cell are detailed in Table 1. Participants with COPD were moderate to severe (with 2 mild and 2 very severe), were significantly older, had a greater prevalence and smoking history, and a higher daily dose of ICS. Participants with asthma had a significantly higher BMI. As expected, FEV_1% predicted and the FEV_1/FVC were significantly lower in asthma subjects than in healthy controls and lower in COPD subjects than in both asthma subjects and healthy controls. Changes in inflammatory cells were present in asthma and COPD, including increases in total cell count, neutrophils, and eosinophils.

3.2. Airway β-Defensin-1 in Asthma Subjects, COPD Subjects, and Healthy Controls. β-defensin-1 protein in sputum was significantly higher in COPD subjects (median (q1 and q3): 63.0 (43.6 and 81.9) ng/mL) than in asthma subjects (26.3 (18.3 and 40.8) ng/mL) and healthy controls (18.2 (14.2 and 27.8) ng/mL; Figure 1). There was no significant difference in β-defensin-1 protein between inflammatory phenotypes of asthma or COPD (Figure 2). β-defensin-1 protein was significantly higher in those with severe asthma (Figure 3) but not different between COPD GOLD stages (data not shown). Airway β-defensin-1 protein level was correlated with smoking history (pack years smoked) in COPD subjects (Spearman $r = 0.52$ and $p = 0.003$, Figure 4) but not in asthma subjects or healthy controls. Airway β-defensin-1 protein level was weakly correlated with ICS dose in asthma (Spearman $r = 0.31$ and $p = 0.006$) but not in COPD, most likely reflecting the relationship to asthma severity. There was no correlation between airway β-defensin-1 protein and age of the participants.

Airway β-defensin-1 protein was predictive of COPD from healthy controls with an accuracy (AUC) of 88.3% (95% CI: 79.3–97.4%; Figure 5(a)). The best cut-off point for β-defensin-1 level to predict COPD from healthy controls was >29.3 ng/mL, with a sensitivity of 87.5%, specificity of 83.3%, and a positive likelihood ratio of 5.3. Airway β-defensin-1 protein was predictive of COPD from asthma with an accuracy (AUC) of 80.8% (95% CI: 72.5–89.1%; Figure 5(b)).

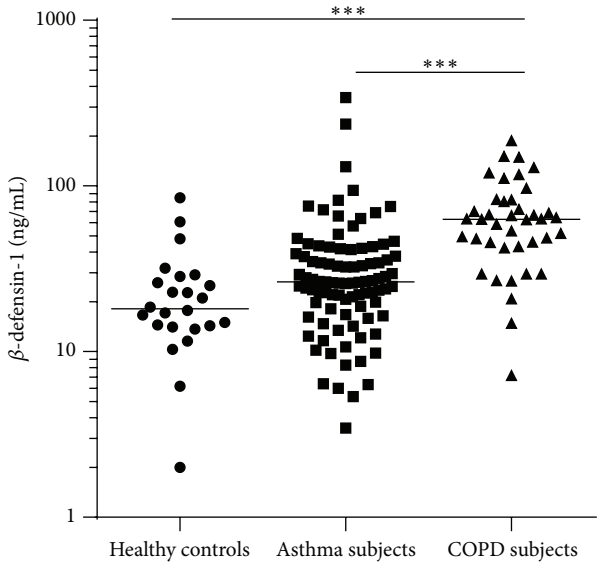

Figure 1: β-defensin-1 in airway disease. The airway expression of β-defensin-1 protein is elevated in participants with COPD compared to asthma patients and healthy controls (Kruskal-Wallis test; $p < 0.001$). The bars represent the median of each group. *** Dunn's post hoc testing $p < 0.001$.

The best cut-off point for β-defensin-1 level to predict COPD from asthma was >42.1 ng/mL, with a sensitivity of 80.0%, specificity of 78.3%, and a positive likelihood ratio of 3.7. Airway β-defensin-1 protein was predictive of COPD from severe asthma with an accuracy (AUC) of 68.7% (95% CI: 55.2–82.2%, Figure 5(c)). The best cut-off point for β-defensin-1 level to predict COPD from severe asthma was >47.3 ng/mL, with a sensitivity of 70.0%, specificity of 72.4%, and a positive likelihood ratio of 2.5.

3.3. Epithelial Cell Production of β-Defensin-1. The level of β-defensin-1 released from untreated media control pBECs was similar between healthy controls and participants with COPD. However, upon stimulation with cigarette smoke extract (CSE) β-defensin-1 production was reduced by a mean of 2.8-fold in healthy control pBECs, whereas COPD pBECs continued to produce the same level of β-defensin-1 (mean −2.8-fold versus 0.1-fold, $p = 0.003$). Figure 6 shows the levels of β-defensin-1 in culture, further illustrating this point, with the COPD group releasing significantly more β-defensin-1 than the healthy control pBECs after 1% CSE stimulation.

4. Discussion

To the authors' knowledge, this was the first study to examine β-defensin-1 protein in sputum in either asthma or COPD. We report an elevated level of β-defensin-1 protein in COPD and severe asthma, with no relationship to inflammatory phenotype. Airway levels of β-defensin-1 were correlated with smoking history in COPD. Airway levels of β-defensin-1 could significantly discriminate the presence of COPD from both asthma subjects and healthy controls. Production of β-defensin-1 was reduced in healthy pBECs but maintained in COPD pBECs after CSE exposure, which may account for the persistent and heightened β-defensin-1 levels.

Factors driving persistent inflammation in asthma and COPD, contributing to worsening of symptoms and lung function, are of interest to increase knowledge of underlying mechanisms and identify potential novel treatment targets. A class of multifunctional antimicrobial proteins known as alarmins, of which the defensins and the cathelicidins constitute two of the major families, may be of importance. Human β-defensin-1 is a small cationic peptide that is expressed constitutively by the epithelial cells of the respiratory tract. Research into the functions of β-defensin-1 has primarily centred around its antimicrobial properties; however other functions have been reported [19]. β-defensin-1 has immunomodulatory effects, promoting the activation and maturation of monocyte derived dendritic cells through upregulation of cell surface expression of costimulatory molecules and maturation markers, as well as the promotion of proinflammatory cytokine production [20]. By engaging a number of cell surface receptors including CCR6, β-defensin-1 promotes the chemotaxis of immature dendritic cells and T cells [21]. Through these immunomodulatory functions, β-defensin-1 may influence the pathogenesis of COPD, by promoting T cell and dendritic cell mediated inflammation.

Polymorphisms in the gene encoding β-defensin-1 (*DEFB1*) have been shown to affect the concentrations of β-defensin-1 protein detected in saliva, indicating that these gene polymorphisms do influence expression and therefore could modify innate immune responses [22], and may influence disease susceptibility. DEFB1 gene polymorphisms have been associated with both asthma [11, 12] and COPD susceptibility [13, 14]. Additionally, increase in *DEFB1* gene expression in bronchial epithelial and BAL fluid cells of patients with COPD is negatively correlated with FEV_1% predicted and FEV_1/FVC [15]. The distinct increase in β-defensin-1 protein seen in the current study supports a model in which β-defensin-1 is dysregulated in COPD and severe asthma. Additionally, we found a weak correlation between β-defensin-1 and ICS dose in asthma. Long term corticosteroid treatment can lead to changes in the balance between innate and adaptive immune responses, including cell migration and the chemokine network in macrophages [23]. Further study is required to determine the effects of long term ICS treatment on β-defensin-1 levels.

The development of new diagnostic tools and treatments for COPD has not kept pace with understanding of the disease [24]. As an airway sample, sputum provides appealing means to study potential biomarkers as it contains a multitude of inflammatory mediators involved in COPD disease processes [25]. This study also shows that β-defensin-1 protein levels in the sputum supernatant may be a useful biomarker for the detection of COPD. β-defensin-1 was able to distinguish COPD from both asthma subjects and healthy controls with high levels of accuracy. For many years, spirometry and clinical symptoms have been the predominant tools to manage COPD; however this approach is not optimal [26]. Sputum eosinophils and exhaled nitric oxide can predict

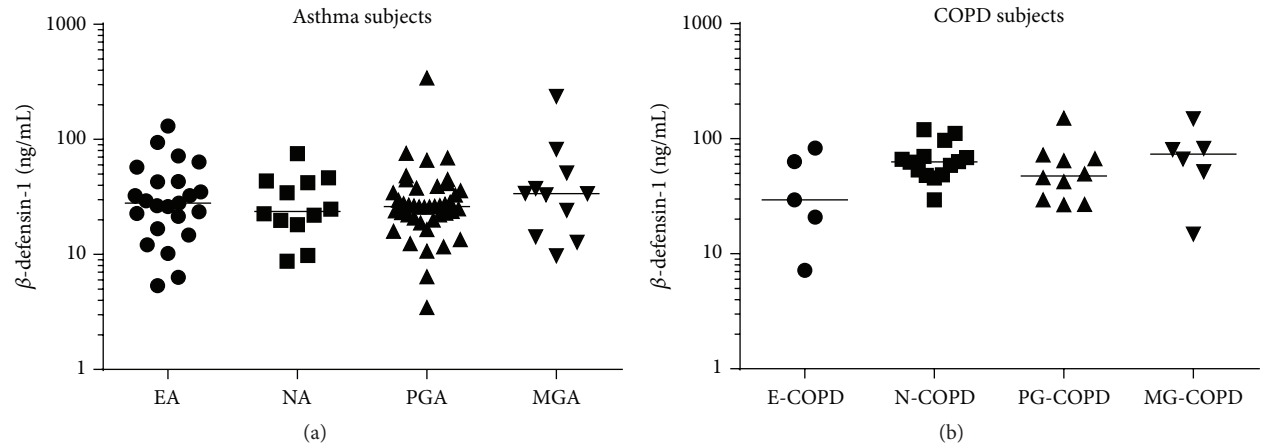

Figure 2: β-defensin-1 in inflammatory phenotypes. The airway expression β-defensin-1 protein is not different between inflammatory phenotypes of (a) asthma and (b) COPD. The bars represent the median of each group. EA = eosinophilic asthma; NA = neutrophilic asthma; PGA = paucigranulocytic asthma; MGA = mixed granulocytic asthma; E-COPD = eosinophilic COPD; N-COPD = neutrophilic COPD; PG-COPD = paucigranulocytic COPD; MG-COPD = mixed granulocytic COPD.

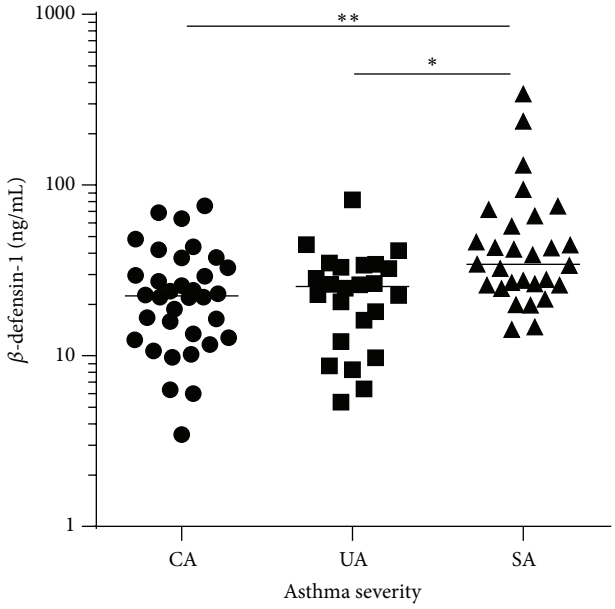

Figure 3: β-defensin-1 and asthma severity. The airway expression of β-defensin-1 protein is higher in severe asthma (SA; $n = 29$) compared with controlled asthma (CA, $n = 34$) and uncontrolled asthma (UA, $n = 24$) (Kruskal-Wallis test; $p = 0.002$). The bars represent the median of each group. *Dunn's post hoc testing $p = 0.003$ versus controlled asthma. **Dunn's post hoc testing $p = 0.015$ versus uncontrolled asthma.

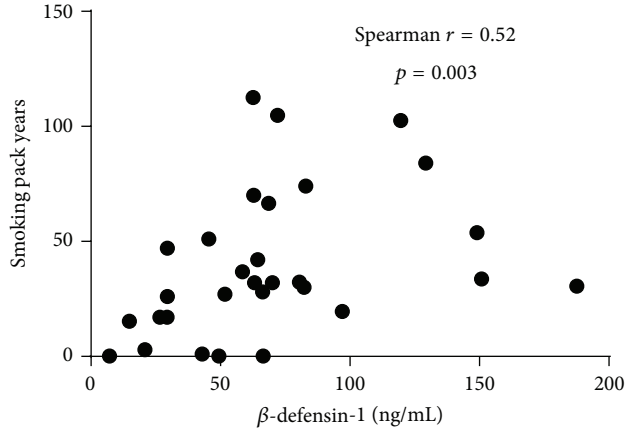

Figure 4: β-defensin-1 and smoking history in COPD. The airway expression of β-defensin-1 protein is correlated with pack years smoked.

eosinophilic inflammation in airways disease [24] but do not differentiate asthma and COPD. Blood biomarkers including C-reactive protein, IL-6, and fibrinogen can detect a systemic inflammatory component of COPD, important in predicting patients at risk of exacerbations [27], but these markers are not specific to COPD and have been determined to be elevated in the neutrophilic asthma phenotype [28]. A plasma protein signature of α_2-macroglobulin, haptoglobin, and hemopexin was shown to discriminate between asthma and COPD with 84% accuracy [29]. This combination of 3 markers was only slightly better than sputum β-defensin-1 alone in the current study at 81%. Follow-up studies are warranted to investigate β-defensin-1 for its capability for predicting COPD diagnosis and prognosis and future risk of exacerbations.

The epithelium is likely the major source of β-defensin-1 that is secreted in the airways. Until recently, only four β-defensins (1, 2, 3, and 4), with their genes clustered on chromosome 8, were known to be expressed by epithelial cells [30]. However, additional β-defensins are predicted to be expressed, though their biological functions are not clear [9]. Regulation of the expression of β-defensin-1 is usually constitutive; however it can be induced in vitro by stimulation with *LPS* and IFN-γ [31], poly I:C [32], bacterial components [33], and TNF-α and IL-1β [34]. β-defensin-2 is induced by bacterial products through activation of toll-like receptors

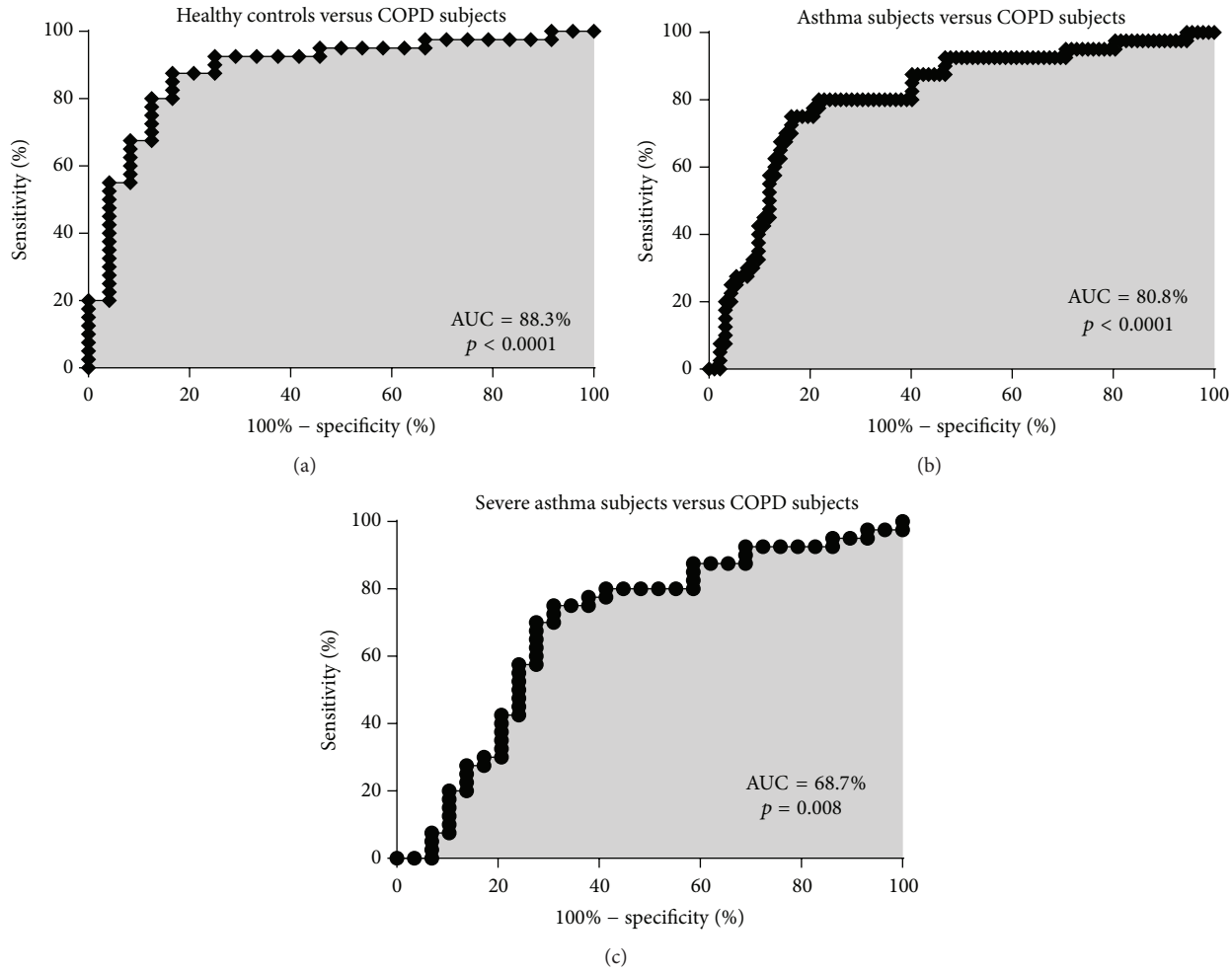

Figure 5: β-defensin-1 as a biomarker. Airway expression of β-defensin-1 can distinguish COPD from (a) healthy controls, (b) asthma patients, and (c) severe asthma patients.

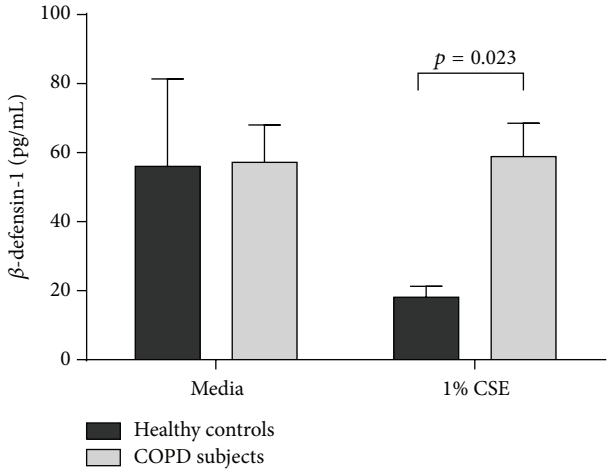

Figure 6: Production of β-defensin-1 from pBECs in response to cigarette smoke extract. The level of β-defensin-1 release is lowered in response to cigarette smoke in healthy pBECs ($n = 4$) but retained in pBECs from subjects with COPD ($n = 10$).

(TLRs) or by proinflammatory cytokines including TNF-α or IL-1β [10]. The expression of β-defensins may therefore produce an environment whereby inflammation is enhanced [10], and there is increased vascular permeability [35]. In this way dysregulation of β-defensin-1 in severe asthma and COPD is likely to be more harmful than beneficial.

Although the production of β-defensins is crucial to proper immune function, altered expression of these molecules may contribute to disease progression. Exposure of the epithelium to cigarette smoke, important in the pathogenesis of COPD, has been shown to influence the production of β-defensins [36]. Our study shows that production of β-defensin-1 was decreased upon exposure to CSE in healthy pBECs; however, when COPD pBECs were exposed, β-defensin-1 production was maintained. This abnormal response of pBECs from COPD patients may be contributing to the higher level of β-defensin-1 seen in the sputum, which may be triggered by exposure to cigarette smoke. Other studies have shown that β-defensin-1 gene expression is also decreased after CSE exposure in A549 cells, a lung alveolar

epithelial cell line [37]. Conversely, other β-defensins are upregulated upon smoke exposure [37, 38]. Further investigation is required to understand the mechanisms causing the abnormal β-defensin-1 production in COPD.

There are a number of limitations to this study. There needs to be further investigation regarding the stimuli responsible for the elevated β-defensin-1 levels in COPD and the mechanisms involved. Future studies should also investigate the relationship of β-defensin-1 and bacterial infection. Given the results of the pBEC culture, we would suspect that the epithelial cells are reprogrammed so that their production of β-defensin-1 does not alter as it should when exposed to different stimuli. We also did not investigate the relationship of β-defensin-1 levels to genotype in this population. Given the associations with DEFB1 polymorphisms and COPD, this should be further investigated. Future studies will need to confirm the biomarker potential of β-defensin-1 in a larger population of subjects with COPD, which will also be necessary for detecting associations with disease severity. Further studies should also investigate the levels of β-defensin-1 in those patients with asthma COPD overlap.

This study shows that β-defensin-1 protein is increased in the airways in COPD and severe asthma but not associated with inflammatory phenotype. In fact, β-defensin-1 expression was a strong biomarker for predicting COPD from both asthma subjects and healthy controls. The level of β-defensin-1 was correlated with smoking history and, in vitro, β-defensin-1 was decreased upon smoke exposure in epithelial cells from healthy participants but maintained in epithelial cells from participants with COPD, suggesting a differential response or tolerance to smoke exposure. This study has identified dysregulated β-defensin-1 production, indicating that this protein may be a therapeutic target for COPD and severe asthma.

Abbreviations

AUC: Area under the curve
CA: Controlled asthma
COPD: Chronic obstructive pulmonary disease
CSE: Cigarette smoke extract
DEFB1: β-defensin-1
FEV_1: Forced expiratory volume in 1 second
FVC: Forced vital capacity
GOLD: Global obstructive lung disease
pBECs: Primary bronchial epithelial cells
ROC: Receiver operating characteristics
SA: Severe asthma
TLR: Toll-like receptor
UA: Uncontrolled asthma.

Disclaimer

Katherine J. Baines is the guarantor and takes the responsibility for the content of this paper, including the data and analysis.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contributions

Study design and conception were done by Katherine J. Baines and Peter G. Gibson. Data collection and interpretation were done by Katherine J. Baines, Thomas K. Wright, Jodie L. Simpson, Vanessa M. McDonald, Lisa G. Wood, Peter A. Wark, Kristy S. Parsons, and Peter G. Gibson. Data analysis was done by Katherine J. Baines and Thomas K. Wright. Writing of the paper was done by Katherine J. Baines and Thomas K. Wright. Editing and review of the paper were done by Katherine J. Baines, Thomas K. Wright, Jodie L. Simpson, Vanessa M. McDonald, Lisa G. Wood, Peter A. Wark, Kristy S. Parsons, and Peter G. Gibson. Guarantor was Katherine J. Baines.

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