Depressive-symptom-associated IL-1β and TNF-α release correlates with impaired bronchodilator response and neutrophilic airway inflammation in asthma

Li Zhang, MD[1, 2*], Xin Zhang, MD[1, 2*], Jing Zheng, MD[3], Ying Liu, MD[1, 2], Ji Wang, MD[1, 2], Gang Wang, MD[1, 4], Hong Ping Zhang, MD[1, 2], De Ying Kang, PhD[5], Zu Gui Peng, MD[6], Yu Lin Ji, MD[7], Lei Wang, MD[1, 2], Peter Gerard Gibson, MBBS[8], Gang Wang, MD, PhD[1, 2, 7]

Running title: Bronchodilator response and inflammation in asthma with depressive symptoms

Abstract word: 299

Manuscript word: 3845

Table and figure count: 6

1. Pneumology Group, Department of Integrated Traditional Chinese and Western Medicine, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, and Collaborative Innovation Center for Biotherapy, Chengdu 610041, P.R. China.

2. Pneumology Group, Department of Integrated Traditional Chinese and Western Medicine, West China Hospital, Sichuan University, Chengdu 610041, P.R. China.

3. Department of Integrated Traditional Chinese and Western Medicine, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, P.R. China.

4. West China School of Medicine, West China Hospital, Sichuan University, Chengdu 610041, P.R. China.

5. Department of Evidence-based Medicine and Clinical Epidemiology, West China

Hospital, Sichuan University, Chengdu 610041, P.R. China.

6. Department of Psychiatry, The Mental Health Center, West China Hospital, Sichuan University, Chengdu 610041, P.R. China.

7. Department of Respiratory and Critical Care Medicine, Clinical Research Center for Respiratory Disease, West China Hospital, Sichuan University, Chengdu 610041, P.R. China.

8. Department of Respiratory and Sleep Medicine, John Hunter Hospital, Hunter Medical Research Institute, University of Newcastle, Priority Research Centre for Healthy Lungs, New Lambton Heights, NSW 2305, Australia.

* These authors contributed equally to this work.

Corresponding author

Dr. Gang Wang, PhD, MD, Department of Respiratory and Critical Care Medicine, Clinical Research Center for Respiratory Disease, West China Hospital, Sichuan University, Chengdu 610041, P. R. China. Tel: +86 28 85422376. E-mail: wcums-respiration@hotmail.com.

Abstract

Background: Depressive symptoms worsen asthma outcomes; however, the mechanism remains largely unexplored.

Objective: This study aimed to determine whether depressive-symptom-associated immune-inflammation correlates with impaired bronchodilator response (BDR) and airway inflammatory phenotypes.

Methods: Eligible adults with asthma (n=198) underwent clinical assessment, sputum induction, and blood sampling. Depressive symptoms were defined by scores on the depression subscale of the Hospital Anxiety and Depression Scale (HADS-D). Pre- and post-bronchodilator spirometry was performed for BDR. Airway inflammatory phenotypes were defined by sputum cell counts. CRP, IL-1β, IL-5, IL-6, IL-8, TNF-α, IFN-γ, CCL17 and CCL22 in serum and sputum were detected.

Results: Compared with the non-depressive group (n=174), the depressive group (n=24) exhibited impaired BDR (P=0.032) and increased sputum neutrophils (P=0.023), which correlated with the HADS-D scores (P=0.027 and P=0.029). Levels of IL-1β, TNF-α and IFN-γ in the serum and those of IL-1β and IFN-γ in the sputum were elevated in the depressive group compared to those in the non-depressive group (all P<0.05). Multiple regression models indicated that TNF-α in the sputum and IL-1β, IL-6 and IFN-γ in both the serum and sputum were inversely associated with BDR; TNF-α in the sputum and IL-1β in both the serum and sputum were positively correlated with sputum neutrophils. Mediation analyses revealed that IL-1β and TNF-α in the sputum and IL-1β in both the serum and sputum mediate the correlations of the HADS-D scores with BDR and sputum neutrophils, respectively.

Conclusions & Clinical Relevance: Asthma patients with depressive symptoms present worse asthma control, which is most likely explained by impaired BDR and neutrophilic airway inflammation. IL-1β and TNF-α, which are two key pro-inflammatory cytokines that mediate the correlation of depressive symptoms with impaired BDR and neutrophilic airway inflammation, may serve as targeted biomarkers in the neuropsychological phenotype of asthma; however, this result needs to be further validated.

Keywords: asthma, depressive symptoms, bronchodilator response, systemic inflammation, airway inflammation phenotype

Introduction

Psychological dysfunction, including depressive symptoms, as a common comorbidity, is significantly associated with uncontrolled asthma and severe, difficult-to-control or glucocorticoid-resistant asthma that leads to more exacerbations, unscheduled or emergency visits and hospitalizations[1,2]. Although this condition may be explained by behaviour, dysfunctional breathing, altered symptom perception, and biological effects induced by psychological dysfunction, the complex mechanisms are still poorly understood[3]. Thus, exploring the underlying mechanisms between psychological factors and asthma would have significant implications for reducing the risk of adverse asthma outcomes, which has been identified as one of the 15 priorities in the roadmap from the European Asthma Research and Innovation Partnership (EARIP)[4]. The precision medicine strategy for asthma has been proposed based on the presence of "treatable traits"[5-7] to obtain a better understanding of controlling asthma. According to the definition of precision medicine[8], psychosocial characteristics including depression problems are important "treatable traits" of the neuropsychological phenotype of asthma, which need to be addressed for the potential implications of asthma precision medicine therapy[7].

Although, until now, identifying psychological dysfunction has been based solely on behavioural symptomatology, increasing evidence indicates crosstalk between the inflammatory pathways and neurocircuits in the brain, which leads to abnormal behavioural responses[9,10]. A recent study has provided the first evidence that peripheral inflammation predates the occurrence of depressive symptoms, as children aged 9 years with high circulating levels of IL-6 were at a 10% greater risk of developing depressive symptoms[11]. Furthermore, depression patients exhibit elevations in inflammation-

related genes, such as *TNFA*, *CRP*, *IL1B*, *IL6*, *TBX21* (*TBET*) and *IFNG*[9,10,12], which could account for the lack of response to approved antidepressant treatments in 30-50% of patients with depression[9]. Our recently published study showed that increased expression of *TBET* or IFN-γ is significantly associated with the Hospital Anxiety and Depression Scale (HADS) scores in asthma patients, which suggests an imbalance in T helper 1 (T_H1)/T_H2 activity towards a predominance of T_H1 response[13]. Furthermore, earlier results for chronic airway diseases have indicated an elevated IFN-γ/IL-5 ratio and IL-6 level in the psychological stress phase[14,15]. All of these inflammatory alterations indicate that inflammation in asthma with depressive symptoms may be different from the T_H2 inflammatory response in classical asthma and that depressive-symptom-associated inflammatory profiles would modify the inflammatory and clinical characteristics of asthma.

Patients suffering from depression exhibit decreased expression of glucocorticoid receptor and β-adrenergic receptor in peripheral blood monocytes, which might potentially contribute to difficulty in treating asthma[16,17]. Recently, it was found that both adult asthma patients with depressive symptoms and asthmatic children with stress exhibit a decreased bronchodilator response (BDR), which is associated with depressive symptoms or stress[18,19]. Furthermore, different sputum cellular phenotypes of asthma exhibit differential response to glucocorticoids, which indirectly reflects the function of the glucocorticoid receptor in the airway[20]. However, the potential immune inflammation underlying the association of depressive symptoms with impaired BDR and airway inflammatory phenotypes in asthma remains unclear.

In this study, we hypothesized that depressive-symptom-associated inflammation such as TNF-α, CRP, IL-1β, IL-6, and IFN-γ was elevated in asthma patients with depressive symptoms, which would be associated with impaired BDR and modified

airway inflammatory phenotypes of asthma. Furthermore, we explored the possible mediation effects of airway and systemic inflammation on the correlation between depressive symptoms and BDR or neutrophilic airway inflammation. Some of the results from this study have been previously presented in the form of an abstract by the American Thoracic Society (ATS) [21].

Materials and methods

Study design and subjects

This was a cross-sectional study based on the Australasian Severe Asthma Network (ASAN)[22]. The ASAN provided the requirements of data collection and sputum induction and performed quality control of the source data.

Adult subjects (≥18 years old) with persistent asthma, which was confirmed via variable airflow obstruction, were recruited proactively and consecutively from the Asthma Clinic of West China Hospital, Sichuan University from March 2014 to February 2016. Variable airflow obstruction was defined as airway hyper-responsiveness in response to any standard challenge agent or positive bronchodilator reversibility test with more than 12% and 200 mL increase of forced expiratory volume in one second (FEV_1) from the baseline[1]. Persistent asthma was required to have no respiratory infection, asthma exacerbation, or change in maintenance therapy in the preceding 4 weeks. The subjects were excluded if they were pregnant or breast feeding, had respiratory diseases other than asthma or chronic diseases of other systems, ever had cardiac or thoracic surgeries or had anxiety symptoms only or cognitive disorder. All participants gave written informed consent, and the Clinical Trial and Biomedicine Ethic Committee in West China Hospital of Sichuan University approved this study (No. 2014-244).

Socio-demographic data, asthma information and sample collection

Socio-demographic data and baseline asthma information including medications and adherence, asthma history, the Asthma Control Questionnaire (ACQ), Asthma Quality of Life Questionnaire (AQLQ), and dyspnoea intensity (Modified Borg Scale,

MBS)[23] were collected. Peripheral venous blood was gathered, and sputum induction and skin-prick testing for atopy were performed as described in our previous studies[22,24].

Depressive symptoms assessment

Depressive symptoms were assessed using the HADS[25]. This assessment contains 7 questions that are specially designed for depressive or anxiety symptoms with a total score of 21 for each. HADS has different sensitivities and specificities in different populations and a cut-off score of 8 could mostly achieve the optimal balance between sensitivity and specificity as both approximate 0.90 for each subscale[25]. In a Chinese population, the HADS has been validated with a sensitivity of 100% and a specificity of 90% for screening of depressive symptoms at a cutoff point of 8[26]. In our study, depressive symptoms only were defined by a HADS depressive symptom (HADS-D) score ≥8 and a HADS anxiety symptom (HADS-A) score <8.

Lung function and bronchodilator response

Spirometry was performed according to the recommendations of the American Thoracic Society/European Respiratory Society recommendations[27]. Short- and long-acting β_2-agonists were demanded to be withheld for at least 24 hours preceding the evaluation[1]. Baseline (pre) and post-salbutamol (post) FEV_1 and forced vital capacity (FVC) were measured using a standardized spirometer (MedGraphics Corp; St. Paul, MN, USA); 400 mcg salbutamol (GSK, A vda de Extremadura, Spain) was administered through adopting a spacer device (150 mL, Wanbo Technology Corp, Shanghai, China). Fifteen minutes later, the post-FEV_1 and post-FVC were measured, and the BDR (change FEV_1, %) was calculated as follows: Change (Δ) FEV_1, % = (post-FEV_1 – pre-FEV_1)/pre-FEV_1 × 100.

Sputum induction and processing

Sputum was induced and processed as described in our previous study[28], and the sputum supernatant and differential cell counts were obtained. Differential cell counts were determined by two well-trained lab researchers from Australia and China. Details are provided in the Methods section in the appendices. Sputum cellular phenotypes were classified as eosinophilic (eosinophils ≥3% and neutrophils <61%), neutrophilic (neutrophils ≥61% and eosinophils <3%), paucigranulocytic (neutrophils <61% and eosinophils <3%) or mixed granulocytic asthma (eosinophils ≥3% and neutrophils ≥61%) based on the presence and absence of sputum granulocytes[29].

Systemic and airway inflammatory cytokine assay

Three panels of cytokines, which were systemic or pro-inflammatory (CRP, TNF-α, IL-1β and IL-6), T_H1/T_H2-like (IFN-γ and IL-5) and M_1/M_2-macrophage-like (TNF-α, IL-1β, IL-6, IL-8, CCL17 and CCL-22) cytokines, were detected both in the serum and sputum supernatant. Serum was obtained from peripheral venous blood that was collected in the Vacutainer tubes (BD Biosciences, San Jose, CA, USA) via centrifugation at 3000 rpm, 4°C for 10 min (H2050R, cence®, Changsha, China). Serum CRP, IL-1β, TNF-α, IFN-γ, IL-5, IL-8, IL-6, CCL22 and CCL17 were quantified via enzyme-linked immunosorbent assay (ELISA) reagent kits (R&D Systems Inc., Minneapolis, USA) following the manufacturer's instructions. Levels of IL-1β, TNF-α, IFN-γ, IL-5, IL-8, IL-6 and CCL22 in the sputum supernatant were measured using the MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel Kit (EMD Millipore Corporation, Billerica, MA, USA) and analysed using the Milliplex Analyst 5.1 software. The concentration was assigned to be half the lower limit concentration

of detection if the cytokine concentration was below the lower limit of detection for the assay[30]. The minimum detectable concentrations of the cytokines in the serum and sputum supernatant are presented in Table E1 in the appendices.

Statistical analysis

The sample size based on BDR was estimated using G*Power version 3.1. According to the incidence of depressive symptoms in asthma, which was approximately 14.05% in the Chinese asthmatic population[31], and the BDR data that were presented in our pre-test study (means of 8.85 and 15.46 for asthma with and without depressive symptoms, respectively, with a pooled standard deviation (SD) of 10.12 at ΔFEV_1 % baseline), a sample size with no fewer than 22 subjects with asthma and depressive symptoms and 132 subjects with asthma only was required for a two-tailed 0.05 level of significance with 80% power[32]. Furthermore, for multiple regressions, we determined the minimum sample size using the formula 50 + 8n, where n represents the number of independent variables[33]. As a result, a sample size of no less than 130 was required in the regression models with 10 independent variables. To allow for 10% withdrawal, we planned to recruit a minimum sample size of 170 subjects, including 25 and 145 asthma subjects with and without depressive symptoms, respectively.

Differences between the groups were evaluated using the t test or the Kruskal-Wallis H test and the Chi-square test or Fisher's exact test, as appropriate. Univariate analyses of Spearman correlation and Linear regression models were applied to explore the relationships between HADS-D scores and BDR, the neutrophil percentage in the sputum, and systemic or airway inflammation. Further mediation analyses were performed according to the method of Baron and Kenny to establish the mediation

effects of systemic or airway inflammatory mediators on the relationship between depressive symptoms and BDR and neutrophil percentage in the sputum[34]. The results of the mediation effects were further confirmed using the Sobel test[35]. The details of the statistical analysis are provided in the Methods section in the supplementary information.

Statistical analyses were performed using SPSS version 21.0 (IBM Corp, Armonk, NY, USA) and a two-tailed *P* value ≤0.05 was considered statistically significant.

Results

Subject characteristics

Two hundred and sixteen asthma subjects were screened; 18 of them with HADS-A scores ≥8 were excluded. Next, the 198 eligible asthma patients were divided into the depressive group (n=24) and the non-depressive group (n=174) based on the cut-off of the HADS-D scores. None of the asthma patients with depressive symptoms received anti-depressant treatments.

Compared with the non-depressive group, the depressive group exhibited worse asthma control as indicated by ACQ (*P*=0.013), worse quality of life as indicated by AQLQ (*P*=0.007) and more serious dyspnoea according to the MBS scores (*P*=0.003) (Table 1). No differences in age, smoking, asthma duration, asthma medications and baseline lung function were observed between the two groups.

Multiple linear regression models indicated that the HADS-D scores were positively associated with the ACQ (β=0.174, *P*=0.038) and MBS (β=0.124, *P*=0.025) scores, while adversely correlated with the AQLQ scores (β=-0.238, *P*=0.003) after adjusting for age, gender, BMI, smoking, asthma duration, ICS dosage and the HADS-A scores.

Bronchodilator response

Airway obstruction, presented as pre- and post-FEV_1, FVC and FEV_1/FVC, exhibited no differences between the depressive and non-depressive groups (Table 2). However, subjects in the depressive group exhibited a significantly decreased BDR in ΔFEV_1 (*P*=0.032 for % and *P*=0.029 for absolute change) in comparison to those in the non-depressive group (Table 2 and Figure 1).

Univariate analyses did not indicate a statistical correlation of the HADS-D scores with ΔFEV_1 (Figure 1); however, it indicated a negative association (β=-0.841, *P*=0.027 for % and β=-0.264, *P*=0.008 for absolute change) with the adjustment of pre-FEV_1% predicted, gender, BMI, smoking, asthma duration, ICS dosage and the HDAS-A scores.

Cellular inflammatory phenotype in sputum

Sputum induction was successfully undertaken in 157 subjects with a median sputum quality score of 14.0 (12.0, 17.0), and 145 of them exhibited better sputum quality (scored >11) to gain sputum cell differential count. In comparison with the non-depressive group, the depressive group exhibited a larger number (*P*=0.014) and a higher percentage (*P*=0.023) of neutrophils, while presenting a lower percentage of macrophages (*P*=0.028) in the sputum (Table 2 and Figure 1). A greater proportion of neutrophilic asthma existed in the depressive group than in the non-depressive group (47.4% vs. 10.2%, *P*<0.001).

The HADS-D scores correlated to the number (r=0.177, *P*=0.038) and the percentage of sputum neutrophils (r=0.204, *P*=0.017) but not that of sputum eosinophils (Figure 1). The multiple regression models also indicated that the HADS-D scores were similarly associated with the sputum neutrophil percentage (β=1.838, *P*=0.029) and the sputum macrophage percentage (β=-2.318, *P*=0.005) after the adjustment for age, gender, BMI, smoking, asthma duration, ICS dosage, asthma exacerbations in the previous year and the HADS-A scores.

Systemic inflammatory cytokines

Serum IL-1β (*P*=0.035), TNF-α (*P*<0.001) and IFN-γ (*P*<0.001) in the depressive group were significantly higher, while the CCL17 concentration (*P*=0.027) was lower

than that in the non-depressive group (Table 3). Serum IL-6 in the depressive group exhibited an elevated trend compared with the non-depressive group (P=0.069). No differences were observed in the CRP, IL-5, IL-8 and CCL22 levels between the two groups.

Serum TNF-α (r=0.259, P=0.021), IFN-γ (r=0.183, P=0.036) and IL-6 (r=0.336, P<0.001) but not CRP (r=0.002, P=0.552) and IL-1β (r=0.165, P=0.094) correlated to the HADS-D scores. After adjusting for age, gender, BMI, smoking, asthma duration, ICS dosage, asthma exacerbations in the previous year and HADS-A scores, our multiple regression models also indicated a positive association of the HADS-D scores with serum IL-1β (β=0.328, P=0.007), TNF-α (β=11.931, P=0.045), IFN-γ (β=6.627, P=0.049) and IL-6 (β=0.377, P=0.046) and a negative association with CCL22 (β=-46.242, P=0.022) and CCL17 (β=-5.932, P=0.047).

Airway inflammatory cytokines

Similar to the systemic inflammation, the depressive group exhibited higher levels of IL-1β (P=0.032) and IFN-γ (P=0.037) in the sputum than the non-depressive group. TNF-α level in the depressive group revealed a tendency of elevation (P=0.063). However, sputum IL-5, IL-8 and CCL22 levels showed no differences between the two groups.

Sputum IL-1β (r=0.195, P=0.020), TNF-α (r=0.181, P=0.031), IFN-γ (r=0.221, P=0.008), but not IL-6 (r=-0.011, P=0.893), were positively associated with HADS-D scores. After adjusting for confounding factors, such as age, gender, BMI, smoking, asthma duration, ICS dosage, asthma exacerbations in the previous year and HADS-A scores, the sputum IL-1β (β=8.407, P=0.044), TNF-α (β=1.988, P=0.015), IFN-γ (β=0.181, P=0.046) and CCL22 (β=-5.401, P=0.026) levels were all associated with

the HADS-D scores. Surprisingly, we found a negative correlation between IL-6 and the HADS-D scores (β=-6.523, *P*=0.006).

Mediation effects of systemic and local inflammation

We performed mediation analyses of systemic and local inflammation in the association of depressive symptoms with BDR and sputum neutrophil percentage. Details are provided in the Methods section in the appendices. Our mediation analyses indicated that sputum IL-1β (β=-0.218, *P*<0.001; Sobel Test: z=-1.970, *P*=0.048) and TNF-α (β=-0.164, *P*=0.003; Sobel Test: z=-1.998, *P*=0.045) were observed to mediate the association between the HADS-D scores and BDR; IL-1β both in the serum (β=51.908, *P*=0.012; Sobel Test: z=1.965, *P*=0.049) and sputum (β=0.382, *P*<0.001; Sobel Test: z=1.962, *P*=0.049) mediated the association of the HADS-D scores with the sputum neutrophil percentage (Table 4).

Discussion

To the best of our knowledge, this is the first study conducted to explore the neuro-immune inflammatory mechanism underlying the relationship of depressive symptoms with BDR and airway cellular inflammatory phenotypes in asthma. This pilot study indicated that asthma patients with depressive symptoms were characterized by worse asthma control and quality of life and more serious dyspnoea that could be explained by impaired BDR and neutrophilic airway inflammation, both of which significantly correlated with depressive symptoms. Furthermore, asthma with depressive symptoms exhibited elevated TNF-α, IL-1β, and IFN-γ, which indicated imbalance of M_1/M_2 and T_H1/T_H2 activities towards a predominance of M_1 and T_H1 response. In addition, mediation analyses indicated that IL-1β and TNF-α, as two important pro-inflammatory cytokines, mediated the association of depressive symptoms with impaired BDR and neutrophilic airway inflammation; these cytokines may serve as targeted biomarkers in the neuropsychological phenotype of asthma.

Consistent with previous studies, asthmatic subjects with depressive symptoms presented worse asthma control in our study[13,36], which could be explained by impaired BDR and neutrophilic airway inflammation. As a heterogeneous disease, asthma is characterized by chronic airway inflammation and bronchodilator reversibility, which were expressed as airway inflammation cell phenotypes and BDR, respectively. Impaired BDR well predicted worse survival advantage in a large population cohort study[37] and correlated with severe exacerbation of asthma in the future[38]. Our study confirmed that depressive symptoms were associated with a 6.52% reduction in BDR, which has also been reported in recently published studies[18]. Furthermore, we found that depressive symptoms were associated with a 13.38% elevation in neutrophils in

the sputum, which indicated a greater incidence of neutrophilic asthma within these patients. It has been shown that neutrophilic inflammation in the airway was responsible for corticosteroid resistance[39] and was mostly associated with severe or refractory asthma[40]. In addition, Shaw *et al.* found that sputum total neutrophil counts were associated with lower post-bronchodilator FEV_1 in asthma[41] perhaps because of the reduced number of mast cells, especially in the $MC_{T/CPA3}$ subtype in the putum[28].

Our study indicated a correlation of elevated IL-1β, TNF-α and IFN-γ, both in the serum and sputum, with the HADS-D scores. In asthmatic populations, IL-1β, TNF-α and IFN-γ are significantly associated with glucocorticoid treatment response and uncontrolled or severe asthma[42-44]. Our previous study also indicated that over-production of IFN-γ would induce MyD88-dependent steroid-resistance in refractory asthma[45]. As an inflammatory disease, in which neuro-immune inflammation and cell-medicated immune activation are involved[9,16,46], major depressive disorder is accompanied by elevated IL-1β, TNF-α and IFN-γ; the subgroup of major depressive disorder patients with increased levels of these cytokines might benefit from an anti-inflammation intervention[47,48]. Psychological stress could induce the activation of pro-inflammatory monocytes in the peripheral blood and lead to an imbalance in the monocyte/macrophage system[16]. Pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ can be secreted by M_1 macrophages, and these cytokines can promote the polarization of T_H1 inflammation[49]. Meanwhile, as a T_H1 inflammatory mediator, IFN-γ can promote M_1 differentiation[50] and induce M_1 macrophages to produce a considerably higher level of IFN-γ via an autocrine feedback mechanism[49]. Thus, M_1 macrophages and T_H1 inflammation may mutually promote to constitute a vicious cycle in the immune inflammatory mechanism of depression. M_2-macrophage-like biomarkers such as CCL22 and CCL17[49], which were inversely associated with the HADS-D scores,

also indicated that M_1 macrophages, as pro-inflammatory cells, may be predominant in this neuropsychological phenotype of asthma[51].

Our further mediation analyses indicated that IL-1β and TNF-α, as two pro-inflammatory cytokines, could play key roles in depressive-symptom-associated impaired BDR and neutrophilic airway inflammation. IL-1β and TNF-α were associated with decreased glucocorticoid expression along with impaired nuclear translocation[52, 53], glucocorticoid receptor phosphorylation[54] and β_2-adrenergic desensitization[55, 56]. Therefore, these results suggest that depressive-symptom-associated activation of systemic M_1 macrophages could produce an over-expression of pro-inflammatory cytokines and promote T_H1 differentiation. These inflammatory mediators may then infiltrate the airway from the peripheral blood and modify the BDR and airway inflammatory phenotype, which could contribute to the development of difficult-to-treat asthma (Figure 2). However, this presumption needs to be further validated in future studies. Additionally, it remains unclear whether there would be an interaction effect of airway and depressive-symptom-associated systemic inflammation.

In this study, we did not observe a significant difference in IL-6 in neither the serum nor the sputum between the depressive and non-depressive subjects with asthma. Interestingly, in our study, serum IL-6 was positively associated with the HADS-D scores, while an opposite association was observed between sputum IL-6 and the HADS-D scores. Reasons for these unexpected findings may be two-fold. Firstly, no significant difference in IL-6 would result from underpowered statistics of biological samples or the depressive symptom as defined by HADS that is used in this study rather than depression diagnosed by Diagnostic and Statistical Manual of Mental Disorder 4th Edition (DSM-IV)[57] that could reduce the internal validity. Secondly, the sources and functions of IL-6 are very complex. As a multifunctional interleukin with both pro- and

anti-inflammatory properties, IL-6 can be produced from diverse cells: it is involved in practically all aspects of the immune system[58], and it plays active roles in the pathogenesis of both asthma and depression[11,48,59,60]. Accordingly, when asthma is present with comorbid depressive symptoms, the status of IL-6 may become much more complex. Until now, few studies have been published to explore the correlation of IL-6 in induced sputum with depressive symptoms. The level of IL-6 in the airway at a local site did not reflect systemic or peripheral regions, which would modify the direction of correlation of sputum IL-6 with depressive symptoms.

Our study has considerable strengths; it exhibited a higher comparability between asthmatics with and without depressive symptoms because of the consecutive recruitment of asthma participants from a real-world setting, in which methods of assessment including lung-function testing and sputum induction and processing were operated via standard procedures by the ASAN programme[22]. In addition, sputum differential cell counts were performed by two well-trained laboratory researchers, and the results exhibited an almost perfect agreement. However, there were still several limitations that have to be addressed. Firstly, the cross-sectional design, paths in the regression models and mediation analyses essentially represented the correlation of depressive symptoms with local and systemic inflammatory biomarkers, BDR, and neutrophilic airway inflammation, which did not indicate the causality. The correlation between asthma and depression is complicated and remains a proverbial "chicken-egg" question until now[3]; however, it may be likely that the presence of pro-inflammatory M_1-like cytokines such as IL-1β and TNF-α appropriately explains this correlation. Secondly, the consecutive sampling method, as one of the non-probability sampling techniques used in our study, is hardly randomized and lacks generalization to some degree; however, a sufficiently large sample size to guarantee statistical power was

adopted in this study to ensure that our resulting sample was more likely to represent the target population than one resulting from simple convenience sampling. Thirdly, the HADS was not a diagnostic tool but only a screening tool for states of depression[61], and further clinical diagnosis of depression was not performed in this study for the patients who screened positively for depressive symptoms, which could strengthen our findings. Importantly, however, the HADS exhibits more than 90% of sensitivity and specificity for diagnosing depression in the Chinese population[26], and it is widely used in the world. . Fourthly, we did not accurately identify which cells produced IL-1β, TNF-α and IFN-γ using flow cytometry, which needs to be explored further.

Conclusions

In conclusion, our study revealed that asthma patients with depressive symptoms were characterized by worse asthma control and quality of life and more serious dyspnoea that could be explained by depressive-symptom-associated impaired BDR and neutrophilic airway inflammation. Asthma patients with depressive symptoms exhibited an elevation of TNF-α, IL-1β, and IFN-γ, which indicated an imbalance of M_1/M_2 and T_H1/T_H2 activities towards a predominance of M_1 and T_H1 response. IL-1β and TNF-α, as two key pro-inflammatory cytokines, mediated the correlation of depressive symptoms with impaired BDR or neutrophilic airway inflammation; these cytokines may be targeted biomarkers in the neuropsychological phenotype of asthma. Our study provides a further understanding of the distinct immune inflammation response in asthma with comorbid depressive symptoms. The neuropsychological phenotype of asthma is unclear and necessitates more studies to explore the complex causalities among depressive symptoms, neuro-immune-mediated inflammation and worse asthma control or severe asthma.

Acknowledgements

We are grateful to Michelle Gleeson and Xiao Fei Liu for their work in the sputum processing.

Funding

This study was supported by the National Natural Science Foundation of China (No. 81570023, 81670023 and 81870027), the National Key Development Plan for Precision Medicine Research (2017YF0910004), Science and Technology Foundation of Sichuan Province (2017SZ1031) and the Program for New Century Excellent Talents in University (no. NCET-12-0380).

Conflict of Interest Statement

The authors declare no conflicts of interest.

References

1. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, Updated 2018. http://www.ginasthma.org.
2. Zhang L, Zhang X, Zheng J, Wang L, Zhang HP, Wang L, et al. Co-morbid psychological dysfunction is associated with a higher risk of asthma exacerbations: a systematic review and meta-analysis. *J Thorac Dis* 2016;**8**:1257-1268.
3. Gerald JK, Moreno FA. Asthma and Depression: It's Complicated. *J Allergy Clin Immunol Pract* 2016;**4**:74-75.
4. Masefield S, Edwards J, Hansen K, Hamerlijnck D, Lisspers K, van der Schee M, et al. The future of asthma research and development: a roadmap from the European Asthma Research and Innovation Partnership (EARIP). *Eur Respir J* 2017;**49**:1602295.
5. Agusti A, Bel E, Thomas M, Vogelmeier C, Brusselle G, Holgate S, et al. Treatable traits: toward precision medicine of chronic airway diseases. *Eur Respir J* 2016;**47**:410-419.
6. Sterk PJ. Chronic diseases like asthma and COPD: do they truly exist? *Eur Respir J* 2016**;47**:359-361.
7. Tay TR, Hew M. Comorbid "treatable traits" in difficult asthma: Current evidence and clinical evaluation. *Allergy* 2018;**73**:1369-1382.
8. Jameson JL, Longo DL. Precision medicine--personalized, problematic, and promising. *N Engl J Med* 2015;**372**:2229-2234.
9. Hodes GE, Kana V, Menard C, Merad M, Russo SJ. Neuroimmune mechanisms of depression. *Nat Neurosci* 2015;**18**:1386-1393.
10. Miller AH, Raison CL. The role of inflammation in depression: from evolutionary imperative to modern treatment target. *Nat Rev Immunol* 2016;**16**:22-34.

11. Khandaker GM, Pearson RM, Zammit S, Lewis G, Jones PB. Association of serum interleukin 6 and C-reactive protein in childhood with depression and psychosis in young adult life: a population-based longitudinal study. *JAMA Psychiatry* 2014;**71**:1121-1128.

12. Jansen R, Penninx BW, Madar V, Xia K, Milaneschi Y, Hottenga JJ, et al. Gene expression in major depressive disorder. *Mol Psychiatry* 2016;**21**:339-347.

13. Zhu M, Liang Z, Wang T, Chen R, Wang G, Ji Y. Th1/Th2/Th17 cells imbalance in patients with asthma with and without psychological symptoms. *Allergy Asthma Proc* 2016;**37**:148-156.

14. Liu LY, Coe CL, Swenson CA, Kelly EA, Kita H, Busse WW. School examinations enhance airway inflammation to antigen challenge. *Am J Respir Crit Care Med* 2002;**165**:1062-1067.

15. Forsythe P, Ebeling C, Gordon JR, Befus AD, Vliagoftis H. Opposing effects of short- and long-term stress on airway inflammation. *Am J Respir Crit Care Med* 2004;**169**:220-226.

16. Carvalho LA, Bergink V, Sumaski L, Wijkhuijs J, Hoogendijk WJ, Birkenhager TK, et al. Inflammatory activation is associated with a reduced glucocorticoid receptor alpha/beta expression ratio in monocytes of inpatients with melancholic major depressive disorder. *Transl Psychiatry* 2014;**4**:e344.

17. Halper JP, Brown RP, Sweeney JA, Kocsis JH, Peters A, Mann JJ. Blunted β-Adrenergic Responsivity of Peripheral Blood Mononuclear Cells in Endogenous Depression. Isoproterenol Dose-Response Studies. *Arch Gen Psychiatry* 1988;**45**:241-244.

18. Han YY, Forno E, Marsland AL, Miller GE, Celedon JC. Depression, Asthma, and Bronchodilator Response in a Nationwide Study of US Adults. *J Allergy Clin*

Immunol Pract 2016;**4**:68-73.

19. Brehm JM, Ramratnam SK, Tse SM, Croteau-Chonka DC, Pino-Yanes M, Rosas-Salazar C, et al. Stress and Bronchodilator Response in Children with Asthma. *Am J Respir Crit Care Med* 2015;**192**:47-56.
20. Gibson PG. Inflammatory phenotypes in adult asthma: clinical applications. *Clin Respir J* 2009;**3**:198-206.
21. Zhang L, Zheng J, Zhang X, Liu Y, Zhang HP, Wang L, et al. Relationships Between Depression and Bronchodilator Response and Airway or Systemic Inflammation in Asthma (abstract). *Am J Respir Crit Care Med* 2017;**195**:A1319.
22. Wang G, Wang F, Gbbson PG, Gou M, Zhang WJ, Gao P, et al. Severe asthma and uncontrolled asthma in China: A survey from the Australasian Severe Asthma Network. *J Thorac Dis* 2017;**9**:1333-1344.
23. Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982;**14**:377-381.
24. Zheng J, Zhang X, Zhang L, Zhang HP, Wang L, Wang G. Interactive effects between obesity and atopy on inflammation: A pilot study for asthma phenotypic overlap. *Ann Allergy Asthma Immunol* 2016;**117**:716-717.
25. Bjelland I, Dahl AA, Haug TT, Neckelmann D. The validity of the Hospital Anxiety and Depression Scale. An updated literature review. *J Psychosom Res* 2002;**52**:69-77.
26. Ye WF, Xu JM. Application and assessment of hospital anxiety and depression scale in general hospital. *Chin J Behavioral Med Sci* 1993;**2**:17-19.
27. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. *Eur Respir J* 2005;**26**:319-338.
28. Wang G, Baines KJ, Fu JJ, Wood LG, Simpson JL, McDonald VM, et al. Sputum

mast cell subtypes relate to eosinophilia and corticosteroid response in asthma. *Eur Respir J* 2016;**47**:1123-1133.

29. Simpson JL, Phipps S, Baines KJ, Oreo KM, Gunawardhana L, Gibson PG. Elevated expression of the NLRP3 inflammasome in neutrophilic asthma. *Eur Respir J* 2014;**43**:1067-1076.

30. Simpson JL, Carroll M, Yang IA, Reynolds PN, Hodge S, James AL, et al. Reduced Antiviral Interferon Production in Poorly Controlled Asthma Is Associated With Neutrophilic Inflammation and High-Dose Inhaled Corticosteroids. *Chest* 2016;**149**:704-713.

31. Li HL, He XL, Liang BM, Zhang HP, Wang Y, Wang G. Anxiety but not depression symptoms are associated with greater perceived dyspnea in asthma during bronchoconstriction. *Allergy Asthma Proc* 2015;**36**:447-457.

32. Dupont WD, Plummer WD, Jr. Power and sample size calculations. A review and computer program. *Control Clin Trials* 1990;**11**:116-128.

33. Tabachnick FB. Multiple regression. In: Using Multivariate Statistics. Boston MA: Allyn & Bacon, 2001.

34. Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. *J Pers Soc Psychol* 1986;**51**:1173-1182.

35. ME S. Direct and indirect effects in linear structural equation models. *Sociol Methods Res* 1987;**15**:151-176.

36. Wang G, Zhou T, Wang L, Wang L, Fu JJ, Zhang HP, et al. Relationship between current psychological symptoms and future risk of asthma outcomes: a 12-month prospective cohort study. *J Asthma* 2011;**48**:1041-1050.

37. Ward H, Cooper BG, Miller MR. Improved criterion for assessing lung function

reversibility. *Chest* 2015;**148**:877-886.

38. Matsunaga K, Hirano T, Oka A, Tanaka A, Kanai K, Kikuchi T, et al. Progression of Irreversible Airflow Limitation in Asthma: Correlation with Severe Exacerbations. *J Allergy Clin Immunol Pract*. 2015;**3**:759-764.e1.

39. Wanderer AA. Proposed pathobiologic mechanisms of hypoxia-ischemia-reperfusion in corticosteroid-resistant neutrophilic asthma and consideration of interleukin 1 targeted therapy. *Ann Allergy Asthma Immunol* 2011;**106**:69-72. doi: 10.1016/j.anai.2010.09.007.

40. Simpson JL, Powell H, Boyle MJ, Scott RJ, Gibson PG. Clarithromycin targets neutrophilic airway inflammation in refractory asthma. *Am J Respir Crit Care Med* 2008;**177**:148-155.

41. Shaw DE, Berry MA, Hargadon B, McKenna S, Shelley MJ, Green RH, et al. Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* 2007;**132**:1871-1875.

42. Chachi L, Abbasian M, Gavrila A, Alzahrani A, Tliba O, Bradding P, et al. Protein phosphatase 5 mediates corticosteroid insensitivity in airway smooth muscle in patients with severe asthma. *Allergy* 2017;**72**:126-136.

43. Chang PJ, Bhavsar PK, Michaeloudes C, Khorasani N, Chung KF. Corticosteroid insensitivity of chemokine expression in airway smooth muscle of patients with severe asthma. *J Allergy Clin Immunol* 2012;**130**:877-885.

44. Hew M, Bhavsar P, Torrego A, Meah S, Khorasani N, Barnes PJ, et al. Relative corticosteroid insensitivity of peripheral blood mononuclear cells in severe asthma. *Am J Respir Crit Care Med* 2006;**174**:134-141.

45. Li JJ, Wang W, Baines KJ, Bowden NA, Hansbro PM, Gibson PG, et al. IL-27/IFN-gamma induce MyD88-dependent steroid-resistant airway

hyperresponsiveness by inhibiting glucocorticoid signaling in macrophages. *J Immunol* 2010;**185**:4401-4409.

46. Maes M. Depression is an inflammatory disease, but cell-mediated immune activation is the key component of depression. *Prog Neuropsychopharmacol Biol Psychiatry* 2011;**35**:664-675.

47. Kopschina Feltes P, Doorduin J, Klein HC, Juárez-Orozco LE, Dierckx RA, Moriguchi-Jeckel CM, et al. Anti-inflammatory treatment for major depressive disorder: implications for patients with an elevated immune profile and non-responders to standard antidepressant therapy. *J Psychopharmacol* 2017;**31**:1149-1165.

48. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A meta-analysis of cytokines in major depression. *Biol Psychiatry* 2010;**67**:446-457.

49. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *Am J Respir Cell Mol Biol* 2015;**53**:676-688. doi: 10.1165/rcmb.2015-0012OC.

50. Zizzo G, Cohen PL. IL-17 stimulates differentiation of human anti-inflammatory macrophages and phagocytosis of apoptotic neutrophils in response to IL-10 and glucocorticoids. *J Immunol* 2013;**190**:5237-5246.

51. Wang L, Liang R, Zhou T, Zheng J, Liang BM, Zhang HP, et al. Identification and validation of asthma phenotypes in Chinese population using cluster analysis. *Ann Allergy Asthma Immunol* 2017;**119**:324-332.

52. Chang PJ, Michaeloudes C, Zhu J, Shaikh N, Baker J, Chung KF, et al. Impaired nuclear translocation of the glucocorticoid receptor in corticosteroid-insensitive airway smooth muscle in severe asthma. *Am J Respir Crit Care Med* 2015;**191**:54-

62.

53. Papi A, Contoli M, Adcock IM, Bellettato C, Padovani A, Casolari P, et al. Rhinovirus infection causes steroid resistance in airway epithelium through nuclear factor kappaB and c-Jun N-terminal kinase activation. *J Allergy Clin Immunol* 2013;**132**:1075-1085.e6.

54. Bouazza B, Krytska K, Debba-Pavard M, Amrani Y, Honkanen RE, Tran J, et al. Cytokines alter glucocorticoid receptor phosphorylation in airway cells: role of phosphatases. *Am J Respir Cell Mol Biol* 2012;**47**:464-473.

55. Rumzhum NN, Patel BS, Prabhala P, Gelissen IC, Oliver BG, Ammit AJ. IL-17A increases TNF-alpha-induced COX-2 protein stability and augments PGE2 secretion from airway smooth muscle cells: impact on beta2 -adrenergic receptor desensitization. *Allergy* 2016;**71**:387-396.

56. Comer BS, Camoretti-Mercado B, Kogut PC, Halayko AJ, Solway J, Gerthoffer WT. Cyclooxygenase-2 and microRNA-155 expression are elevated in asthmatic airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2015;**52**:438-447.

57. American Psychiatric Association. Diagnostic and statistical manual of mental disorders (4th ed). Washington, DC: American Psychiatric Association; 1994.

58. Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. *Nat Immunol* 2015;**16**:448-57.

59. Ferreira MA, Matheson MC, Duffy DL, Marks GB, Hui J, Le Souëf P, et al. Identification of IL6R and chromosome 11q13.5 as risk loci for asthma. *Lancet* 2011;**378**:1006-1014.

60. Peters MC, McGrath KW, Hawkins GA, Hastie AT, Levy BD, Israel E, et al. Plasma interleukin-6 concentrations, metabolic dysfunction, and asthma severity: a cross-sectional analysis of two cohorts. *Lancet Respir Med* 2016;**4**:574-584.

61. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983;**67**:361-370.

Table 1. Characteristics of asthma participants with depressive and non-depressive symptoms.

Variables	Non-depressive group	Depressive group	*P*
n	174	24	
Age	47.28±13.79	44.45±9.71	0.214
Gender, male/female	70/104	11/13	0.601
BMI	23.71±3.91	24.19±4.37	0.574
Smoking status			
Current/ex-/non-smoker	21/29/124	5/6/13	0.228
Pack years	0.00 (0.00, 3.71)	0.00 (0.00, 9.32)	0.370
Atopy, n (%)	95 (54.6)	17 (70.8)	0.133
HADS-D scores	1.0 (0.0, 3.0)	9.0 (8.0, 10.0)	<0.001
HADS-A scores	2.0 (0.0, 3.0)	5.5 (4.0, 6.0)	<0.001
Asthma duration, years	7.66 (2.65, 22.44)	8.65 (2.48, 16.48)	0.789
Asthma exacerbations in the previous year	0 (0, 0)	0 (0, 1)	0.225
ACQ	0.67 (0.17, 1.33)	1.33 (0.50, 2.12)	0.013
AQLQ	6.11 (5.47, 6.53)	5.55 (4.72, 6.28)	0.007
Modified Borg Scale scores	1.0 (0.0, 3.0)	2.00 (2.00, 4.00)	0.003
Asthma medications			
ICS and LABA, n (%)	72 (41.4)	9 (37.5)	0.717
ICS dosage (BPD), μg	400 (400, 1000)	400 (400, 400)	0.310
Leukotriene modifier, n (%)	40 (23.0)	7 (29.2)	0.505
Theophylline, n (%)	33 (19.0)	6 (25.0)	0.486
LAMA, n (%)	4 (2.3)	1 (4.2)	0.585
Adherence to ICS, %	88.9	73.6	0.440
GINA treatment steps, steps 1-3/4-5	116/58	16/8	1.000
Baseline lung function (pre β_2 agonist)			
FEV_1, L	2.15±0.78	2.26±0.75	0.526
FEV_1, % predicted	74.03±19.75	75.84±21.52	0.678
FEV_1/FVC, %	67.08±13.84	68.32±13.28	0.680

ACQ: Asthma Control Questionnaire; AQLQ: Asthma Quality of Life Questionnaire; BMI: body mass index; BPD: beclomethasone dipropionate; GINA: Global Initiative for Asthma; HADS-D: depressive symptoms of the Hospital Anxiety and Depression Scale; HADS-A: anxiety symptoms of the Hospital Anxiety and Depression Scale; ICS: inhaled corticosteroid; LABA: long-acting beta2-agonist; LAMA: long-acting anticholinergic drugs.

Table 2. Bronchodilator response and sputum cellularity of asthma patients grouped by depressive symptoms.

Variables	Non-depressive group	Depressive group	*P*
n	174	24	
Lung function, pre β_2 agonist			
FEV_1, L	2.15±0.78	2.26±0.75	0.526
FEV_1, % predicted	74.03±19.75	75.84±21.52	0.678
FVC, L	3.19±0.93	3.301±1.04	0.576
FVC, % predicted	91.59±16.58	91.72±18.42	0.973
FEV_1/FVC, %	67.08±13.84	68.32±13.28	0.680
Lung function, post β_2 agonist			
FEV_1, L	2.36±0.82	2.43±0.66	0.719
FEV_1, % predicted	81.07±17.91	79.00±18.45	0.656
FVC, L	3.38±0.93	3.43±0.66	0.806
FVC, % predicted	96.88±14.44	92.99±16.87	0.307
FEV_1/FVC, %	69.41±13.06	71.38±12.91	0.558
Δ FEV_1, L	0.30±0.21	0.20±0.14	0.029
Δ FEV_1, %	16.72±10.77	10.20±7.28	0.032
Sputum cell differential count			
n	126	19	
Total cell count, $\times 10^6$/L	2.49 (1.30, 4.21)	4.05 (1.58, 7.34)	0.166
Neutrophils, $\times 10^6$/L	0.70 (0.18, 1.58)	0.87 (0.22, 2.83)	0.014
Neutrophil, %	32.38 (11.88, 46.56)	45.50 (9.50, 80.50)	0.023
Eosinophils, $\times 10^6$/L	0.00 (0.00, 0.03)	0.00 (0.00, 0.01)	0.356
Eosinophil, %	0.00 (0.00, 1.00)	0.00 (0.00, 0.50)	0.289
Macrophages, $\times 10^6$/L	1.46 (0.84, 2.65)	0.86 (0.51, 2.72)	0.330
Macrophage, %	62.75 (45.94, 84.63)	34.00 (18.75, 81.50)	0.028
Lymphocytes, $\times 10^6$/L	0.02 (0.01, 0.06)	0.02 (0.01, 0.07)	0.963
Lymphocyte, %	1.00 (0.50, 2.00)	0.75 (0.50, 2.00)	0.747

Δ: change from the baseline.

Table 3. Systemic and airway inflammatory cytokines in asthma patients with and without depressive symptoms.

Variables	Non-depressive group	Depressive group	*P* values
Serum			
n	174	24	
CRP, pg/mL	1.18 (1.00, 3.84)	1.00 (1.00, 2.87)	0.570
IL-1β, pg/mL	0.50 (0.50, 0.50)	0.50 (0.50, 0.67)	0.035
TNF-α, pg/mL	15.00 (15.00, 15.00)	15.00 (15.00, 225.54)	<0.001
IFN-γ, pg/mL	19.69 (3.62, 57.88)	21.73 (3.25, 243.24)	<0.001
IL-5, pg/mL	2.92 (0.15, 16.29)	12.94 (4.26, 22.16)	0.183
IL-6, pg/mL	1.62 (1.50, 4.55)	3.84 (1.50, 7.29)	0.069
IL-8, pg/mL	20.81 (13.92, 25.34)	17.97 (11.55, 25.96)	0.688
CCL22, pg/mL	87.15 (60.87, 492.90)	68.90 (49.31, 130.32)	0.203
CCL17, pg/mL	120.00 (64.51, 231.80)	65.94 (35.20, 151.23)	0.027
Sputum			
n	135	22	
IL-1β, pg/mL	18.01 (8.45, 39.25)	39.29 (13.54, 130.65)	0.032
TNF-α, pg/mL	12.49 (4.45, 28.27)	20.46 (5.49, 68.07)	0.063
IFN-γ, pg/mL	1.12 (0.40, 1.68)	1.52 (1.10, 2.10)	0.037
IL-5, pg/mL	1.40 (0.91, 2.23)	0.95 (0.79, 2.02)	0.149
IL-6, pg/mL	20.75 (8.38, 52.83)	41.61 (16.52, 77.91)	0.156
IL-8, pg/mL	1791.00 (802.53, 3521.75)	2531.00 (980.58, 3949.00)	0.360
CCL22, pg/mL	49.98 (23.33, 97.96)	33.92 (20.85, 72.06)	0.191

Table 4. Mediation effects validated by Sobel tests.

Inflammatory mediators	Correlation between HADS-D scores and BDR			Correlation between HADS-D scores and sputum neutrophils (%)		
	z value	SE	*P*	z value	SE	*P*
Cytokines in serum						
CRP, pg/mL	-0.076	0.008	0.938	0.078	0.045	0.937
IL-1β, pg/mL	-1.187	1.811	0.235	1.965	8.663	0.049
TNF-α, pg/mL	-1.034	0.138	0.300	-1.138	0.503	0.255
IFN-γ, pg/mL	-1.072	0.086	0.283	-0.999	0.245	0.317
IL-5, pg/mL	-0.443	0.203	0.657	-0.557	0.343	0.576
IL-6, pg/mL	-1.042	0.100	0.297	1.250	0.244	0.211
IL-8, pg/mL	-0.070	0.007	0.943	0.070	0.050	0.943
CCL22, pg/mL	1.516	0.121	0.129	0.682	0.338	0.494
CCL17, pg/mL	0.110	0.053	0.911	0.581	0.173	0.561
Cytokines in sputum						
IL-1β, pg/mL	-1.970	0.930	0.048	1.962	1.636	0.049
TNF-α, pg/mL	-1.998	0.163	0.045	1.525	0.177	0.127
IFN-γ, pg/mL	-1.195	0.176	0.231	-1.308	0.436	0.190
IL-5, pg/mL	0.119	0.010	0.904	-0.144	0.058	0.885
IL-6, pg/mL	1.355	0.081	0.175	0.038	0.169	0.969
IL-8, pg/mL	0.000	0.002	1.000	-0.821	0.002	0.411
CCL22, pg/mL	0.453	0.070	0.878	-0.032	0.167	0.974

FEV_1: forced expiratory volume in 1 second; HADS-D: depressive symptom of the Hospital Anxiety and Depression Scale; Δ: change from the baseline.

Figure legends

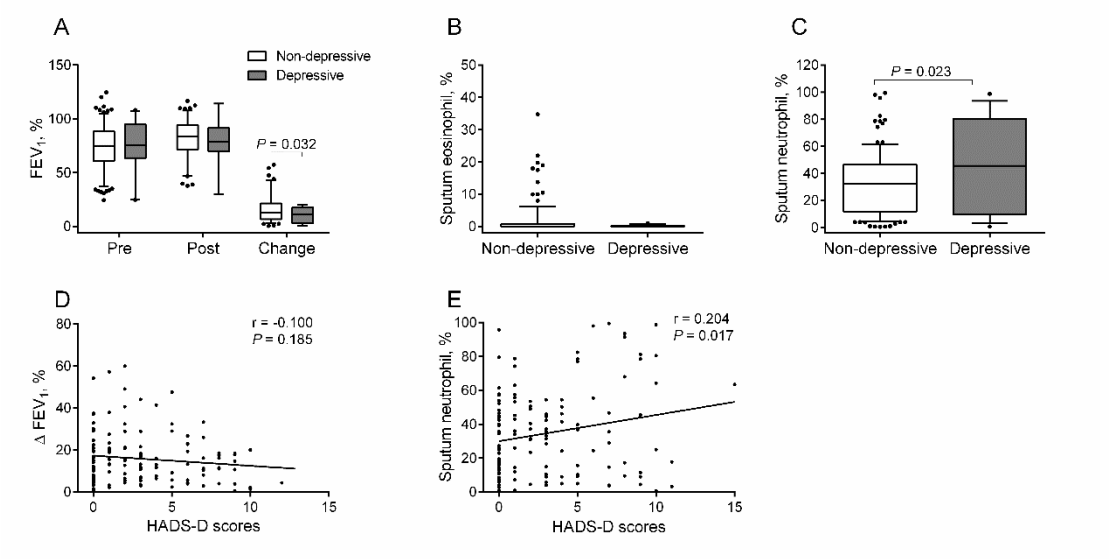


Figure 1. Bronchodilator response (A) and sputum eosinophils (B) and neutrophils (C) in asthma patients with and without depressive symptoms, and the correlations of depressive symptoms in HADS-D scores with bronchodilator response (D) and sputum neutrophils (E). HADS-D: depressive symptom of the Hospital Anxiety and Depression Scale; Δ: change from the baseline. Data are expressed as the median (quartile) with 5-95 percentile.

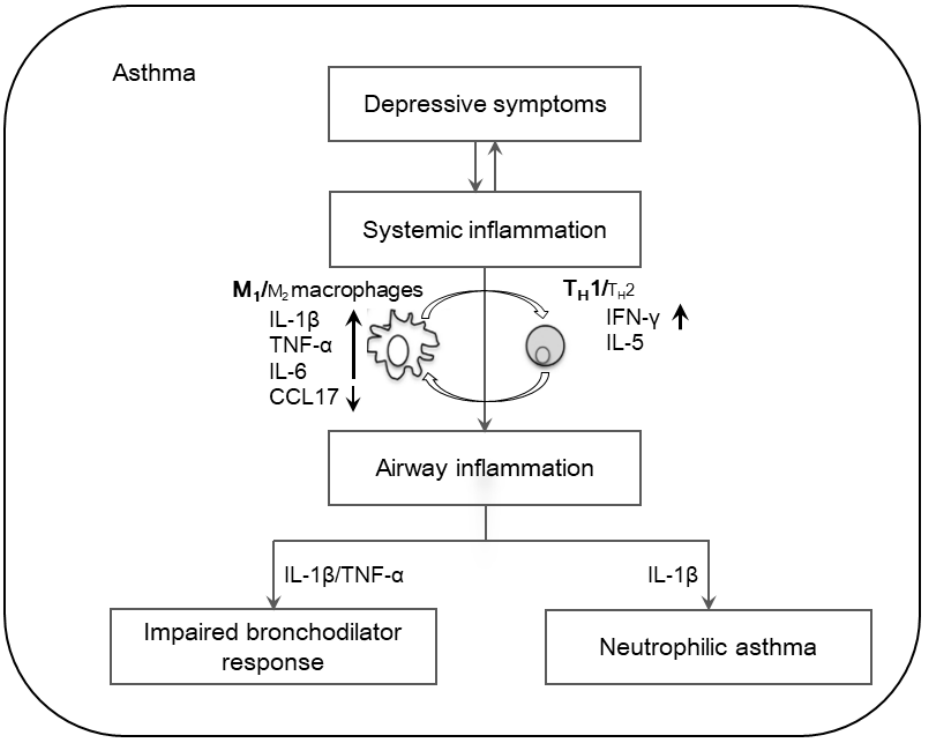


Figure 2. Hypothetic schematic diagram for the mechanism underlying the correlation of depressive symptoms with impaired bronchodilator response and neutrophilic airway inflammation in asthma with depressive symptoms.

Appendices

Depressive-symptom-associated IL-1β and TNF-α release correlates with impaired bronchodilator response and neutrophilic airway inflammation in asthma

Li Zhang, Xin Zhang, Jing Zheng, Ying Liu, Ji Wang, Gang Wang, Hong Ping Zhang, De Ying Kang, Zu Gui Peng, Yu Lin Ji, Lei Wang, Peter Gerard Gibson, Gang Wang

Materials and methods

Sputum induction and processing

Sputum was induced after pretreatment with 400 mcg salbutamol (GSK, Avda de Extremadura, Spain) using 4.5% saline that was atomized using an ultrasonic nebulizer (Cumulus, HEYER Medical AG, German). If the pre- or post-FEV_1 was ≤40% of the predicted value, the sputum was induced with 0.9% saline after it was deemed safe by the supervising physician.

The volume of the selected sputum plugs was determined. A volume of 1% dithiothreitol (SPUTOLYSIN Reagent, Calbiochem®, USA) was added equal to 4 times the sputum volume. The sputum sample was gently mixed using a rotating mixer and placed in a shaking water bath at room temperature for approximately 30 min to ensure complete homogenization. The homogenized sample, with the addition of a volume of PBS as sputolysin, was mixed with a disposable pipette and shaken for further 5 min, filtered through the wet nylon filter apparatus, and centrifuged at 1500

824 rpm for 10 min (H2050R, cence®, Changsha, China). Finally, the sputum supernatant
825 was aspirated and frozen immediately at -80°C for further analysis. PBS was added to
826 the cell pellets that were obtained after centrifugation, and the cells were suspended.
827 Total and differential cell counts were obtained via centrifugation-smear (CYTOPRO
828 7620, WESCOR®, INC., Logan, USA) and staining (Giemsa, SIGMA-ALDRICHTM,
829 Inc., Louis, USA) preparation. To achieve better differential cell counts, the sputum
830 quality was assessed using the cytospin quality scale from Hunter Medial Research
831 Institute, Australia (Table E2). In addition, differential cell counts in our study were
832 determined by two well-trained laboratory researchers, Michelle Gleeson in Hunter
833 Medical Research Institute, Australia and Xiao Fei Liu at West China Hospital, China.
834 The agreement analysis between the observers showed a high agreement from 80.33%
835 to 100.00% for the overall data (Kappa=0.901, P<0.001) and different cell types in
836 neutrophils with an agreement of 96.24% (Kappa=0.921, P<0.001), eosinophils with
837 an agreement of 100.00% (Kappa=1.000, P<0.001), macrophages with an agreement
838 of 94.81% (Kappa=0.891, P<0.001), and lymphocytes with an agreement of 97.50%
839 (Kappa=0.728, P<0.001) (Table E3).
840
841 **Statistical analysis**
842 Continuous variables were expressed as the means and SD for normal distribution
843 or as the medians and interquartile range (IQR) for abnormal distribution respectively.
844 Categorical variables were expressed as the percentages. The t test or the Kruskal-
845 Wallis H test was performed for continuous data, and the Chi-square test or Fisher's

exact test was used for categorical data analysis.

Linear regression models were applied to explore the relationships between the HADS-D scores and BDR or neutrophil percent in the sputum and the systemic or airway inflammation. Univariate analyses were initially conducted using the Spearman correlation; any variable significant at P <0.20 without collinearity to each other was entered into the multiple regression models. In addition, potential variables affecting BDR, sputum cellular phenotypes or systemic and airway inflammation, including age, gender, body mass index (BMI), smoking, FEV_1 % predicted, asthma duration, inhaled corticosteroids (ICS) dosage and HADS-A scores were also included in the models as priori covariates regardless of P-value. The results of the models were presented as beta coefficients (β), standard error (SE) and 95% confidence interval (CI).

Mediation analyses were performed step by step following the methods by Baron and Kenny, which contains four steps of multiple regression to explore the mediation effects of systemic or airway inflammatory mediators on the relationships between depressive symptom and BDR or neutrophil percentage in sputum[1,2]. For example, as described in Figure E1, BDR was correlated with depressive symptoms (path c in Figure E1 A). The association between depressive symptoms and BDR (path c′) was mediated by an increase in inflammatory mediators which was associated with depressive symptoms (path a) and was responsible for BDR (path b', when the depressive symptoms were also a predictor of the BDR) (Figure E1 B). Separate coefficients (β) for each equation should be estimated and tested; β_1, β_2, and β_3 were path coefficients. We also confirmed the mediation results using the Sobel test[3].

868

Results

Mediation effects of systemic and local inflammation

Mediation analysis was performed step by step to explore the possible mediation effects of local and systemic inflammatory mediators on the correlations between depressive symptoms, which were represented by HADS-D scores, and BDR or the percentage of sputum neutrophils. First, we examined the correlations between the HADS-D scores and BDR or the percentage of sputum neutrophils and local or systemic inflammation mediators. As described in the main document, the HADS-D scores were significantly associated with BDR (β=-0.841, *P*=0.027) and the percentage of sputum neutrophils (β=1.838, *P*=0.029) (Table E4). In term of the associations between HADS-D scores and local and systemic inflammation, HADS-D scores correlated with serum IL-1β (β=0.328, *P*=0.007), TNF-α (β=11.931, *P*=0.045), IFN-γ (β=6.627, *P*=0.049), IL-6 (β=0.377, *P*=0.046), CCL22 (β=-46.242, *P*=0.022), CCL17 (β=-5.932, *P*=0.047) and sputum IL-1β (β=8.407, *P*=0.044), TNF-α (β=1.988, *P*=0.015), IFN-γ (β=0.181, *P*=0.046), IL-6 (β=-6.523, *P*=0.006), CCL22 (β=-5.401, *P*=0.026) (Table E4). It further indicated that serum IL-1β (β=-9.627, *P*=0.041), IFN-γ (β=-0.164, *P*=0.050) and IL-6 (β=-0.423, *P*=0.040) and sputum IL-1β (β=-0.231, *P*=0.006), TNF-α (β=-0.158, *P*=0.046), IFN-γ (β=-1.464, *P*=0.048), and IL-6 (β=-0.162, *P*=0.036) were all significantly associated with BDR; serum IL-1β (β=30.508, *P*=0.032) and IL-6 (β=1.225, *P*=0.016) and sputum IL-1β (β=0.412, *P*<0.001) and TNF-α (β=0.151,

889 *P*=0.028) were significantly correlated with the percentage of sputum neutrophils
890 (Table E5).

891 Second, we sought to explore the combined effects of depressive symptom scores
892 and inflammatory mediators on BDR or the percentage of sputum neutrophils to
893 determine the potential mediation effects of systemic or local inflammation on the
894 correlations between depressive symptoms and BDR or asthma inflammatory
895 phenotypes (Table E6). The Sobel test was further performed to confirm these
896 mediation effects. As a result, sputum IL-1β (β=-0.218, *P*<0.001) and TNF-α (β=-0.164,
897 *P*=0.003) were observed to mediate the associations between the HADS-D scores and
898 BDR, and IL-1β both in the serum (β=51.908, *P*=0.012) and sputum (β=0.382, *P*<0.001)
899 was observed to be a mediator of the correlation between the HADS-D scores and the
900 percentage of sputum neutrophils (Table E6). Finally, the following Sobel test also
901 confirmed the significant mediation effects of serum IL-1β (z=1.965, *P*=0.049 for the
902 percentage of sputum neutrophils), sputum IL-1β (z=-1.970, *P*=0.048 for BDR and
903 z=1.962, *P*=0.049 for the percentage of sputum neutrophils) and sputum TNF-α (z=-
904 1.998, *P*=0.045 for BDR).
905

References

E1. Tabachnick FB. Multiple regression. In: Using Multivariate Statistics. Boston: MA: Allyn & Bacon; 2001.E5. Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. *J Pers Soc Psychol* 1986;**51**:1173-1182.

E2. Boudreau M, Bacon SL, Ouellet K, Jacob A, Lavoie KL. Mediator effect of depressive symptoms on the association between BMI and asthma control in adults. *Chest* 2014;**146**:348-354.

E3. ME S. Direct and indirect effects in linear structural equation models. *Sociol Methods Res* 1987;**15**:151-176.

Table E1. Minimum detectable concentrations of the cytokines.

Cytokines	Minimum detectable concentration, pg/mL	
	ELISA for serum	MILLIPLEX for SP
CRP	2.0	-
IL-1β	1.0	1.2
TNF-α	15.6	0.7
IFN-γ	8.0	0.8
IL-5	0.29	0.5
IL-8	7.5	0.4
IL-6	0.7	0.9
CCL22	62.5	3.6
CCL17	7.0	-

ELISA: enzyme-linked immunosorbent assay; SP: sputum supernatant.

920 Table E2. Items of sputum cytospin quality.

Items	Descriptors and scores	Scoring
Debris	nil (4); scant (3); moderate (2); excessive (1)	
Cell outline	preserved (4); isolated cell damage (3); many cells damaged (2); most cells damaged (1)	
Nuclear morphology	preserved (4); isolated nuclei damage (3); many nuclei damaged (2); most nuclei damaged (1)	
Squamous	< 20% (4); 21–60% (3); 61–85% (2); > 85% (1)	
Overall impression	good (4); acceptable (3); just acceptable (2); bad (1)	
Slide macrophages present	yes (1); no (0)	
Number of cells on each slide	≥ 400 (2); 200-399 (1); ≤ 200 (0)	
Total scores		

921

922

923 Table E3. Agreement analysis for sputum cell-count data between observers from Australia and China.

Batches	Agreement (%)	Kappa	*P*
1	80.33	0.722	< 0.001
2	95.24	0.924	< 0.001
3	100.00	1.00	< 0.001
4	83.13	0.700	< 0.001
5	95.71	0.933	< 0.001
6	98.89	0.983	< 0.001
7	96.67	0.950	< 0.001
8	96.08	0.929	< 0.001
Total	93.38	0.901	< 0.001
Cell types			
Neutrophils	96.24	0.921	< 0.001
Eosinophils	100.00	1.000	< 0.001
Macrophages	94.81	0.891	< 0.001
Lymphocytes	97.50	0.728	< 0.001

924

Table E4. Associations between depressive symptoms scores and bronchodilator response, sputum neutrophil percentage or inflammatory mediators.

Variables	HADS-D scores		
	β_2	SE_2	*P*
Δ FEV_1, % #	-0.841	0.375	0.027
Sputum neutrophils, % *	1.838	0.829	0.029
	β_1	SE_1	*P*
Cytokines in serum *			
CRP, pg/mL	0.038	0.482	0.937
IL-1β, pg/mL	0.328	0.117	0.007
TNF-α, pg/mL	11.931	5.849	0.045
IFN-γ, pg/mL	6.627	3.328	0.049
IL-5, pg/mL	0.026	3.151	0.883
IL-6, pg/mL	0.377	0.187	0.046
IL-8, pg/mL	0.040	0.562	0.944
CCL22, pg/mL	-46.242	19.876	0.022
CCL17, pg/mL	-5.932	2.961	0.047
Cytokines in sputum supernatant *			
IL-1β, pg/mL	8.407	4.128	0.044
TNF-α, pg/mL	1.988	0.807	0.015
IFN-γ, pg/mL	0.181	0.091	0.046
IL-5, pg/mL	3.623	0.177	0.258
IL-6, pg/mL	-6.523	2.309	0.006
IL-8, pg/mL	-2.234	1.551	0.973
CCL22, pg/mL	-5.401	2.396	0.026

HADS-D: depressive symptoms of the Hospital Anxiety and Depression Scale; SE: standard error.
#: This model was adjusted for pre-FEV_1, % predicted; gender; body mass index; smoking; asthma duration; inhaled corticosteroid daily dosage; asthma exacerbations in the previous year; and anxiety symptom scores of the Hospital Anxiety and Depression Scale.
*: All models were adjusted for age, gender, body mass index, smoking, asthma duration, inhaled corticosteroid dosage, asthma exacerbations in the previous year and anxiety symptom scores on the Hospital Anxiety and Depression Scale.

Table E5. Associations of inflammatory mediators with bronchodilator response and sputum neutrophil percentage.

Variables	Δ FEV_1, % *			Sputum neutrophils, % #		
	β_3	SE_3	*P*	β_3	SE_3	*P*
Cytokines in serum						
CRP, pg/mL	-0.019	0.054	0.723	0.119	0.166	0.475
IL-1β, pg/mL	-9.627	4.609	0.041	30.508	13.946	0.032
TNF-α, pg/mL	-0.017	0.009	0.074	-0.021	0.030	0.482
IFN-γ, pg/mL	-0.164	0.082	0.050	-0.031	0.029	0.286
IL-5, pg/mL	-0.046	0.054	0.392	0.015	0.069	0.825
IL-6, pg/mL	-0.423	0.203	0.040	1.225	0.496	0.016
IL-8, pg/mL	-0.016	0.026	0.525	0.082	0.084	0.335
CCL22, pg/mL	-0.004	0.002	0.077	-0.004	0.007	0.556
CCL17, pg/mL	0.001	0.009	0.947	-0.025	0.029	0.378
Cytokines in sputum supernatant						
IL-1β, pg/mL	-0.231	0.087	0.006	0.412	0.090	<0.001
TNF-α, pg/mL	-0.158	0.078	0.046	0.151	0.068	0.028
IFN-γ, pg/mL	-1.464	0.730	0.048	-2.796	1.832	0.130
IL-5, pg/mL	0.044	0.235	0.852	-0.369	0.418	0.379
IL-6, pg/mL	-0.162	0.072	0.0**36**	-0.002	0.027	0.953
IL-8, pg/mL	0.00	0.001	0.766	0.001	0.001	0.306
CCL22, pg/mL	0.001	0.013	0.958	0.002	0.029	0.938

HADS-D: depressive symptoms of the Hospital Anxiety and Depression Scale; β: regression coefficient; SE: standard error.

*: All models were adjusted for pre-FEV_1, % predicted; gender; body mass index; smoking; asthma duration; and inhaled corticosteroid daily dosage.

#: All models were adjusted for age, gender, body mass index, smoking, asthma duration and inhaled corticosteroid dosage.

Table E6. The effects of depressive symptoms on bronchodilator response and neutrophil percentage in sputum mediated by inflammatory mediators.

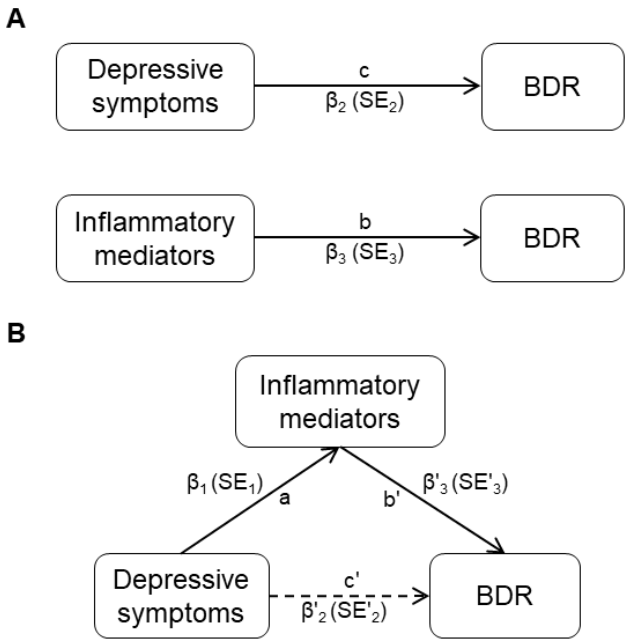
	Path b': Inflammatory mediators						Path c': HADS-D scores					
	Δ FEV_1, % *			Sputum neutrophils, % #			Δ FEV_1, % *			Sputum neutrophils, % #		
Variables	β_3'	SE_3'	*P*	β_3'	SE_3'	*P*	β_2'	s_2'	*P*	β_2'	s_2'	*P*
Cytokines in serum												
CRP, pg/mL	-0.018	0.052	0.733	0.093	0.166	0.578	-0.060	0.411	0.884	1.333	1.103	0.231
IL-1β, pg/mL	-6.599	5.003	0.192	51.908	18.837	0.012	-0.296	0.486	0.544	-1.030	1.125	0.364
TNF-α, pg/mL	-0.012	0.010	0.274	-0.048	0.035	0.180	-0.422	0.656	0.524	3.151	1.803	0.089
IFN-γ, pg/mL	-0.014	0.011	0.207	-0.037	0.032	0.242	-0.225	0.477	0.638	1.588	1.045	0.133
IL-5, pg/mL	-0.025	0.052	0.641	-0.053	0.083	0.530	-0.338	0.685	0.625	3.008	1.993	0.140
IL-6, pg/mL	-0.279	0.229	0.226	0.811	0.509	0.115	0.108	0.380	0.778	1.147	1.047	0.277
IL-8, pg/mL	-0.013	0.028	0.651	0.089	0.090	0.331	-0.177	0.604	0.771	1.423	1.525	0.356
CCL22, pg/mL	-0.004	0.002	0.125	-0.005	0.007	0.482	-0.469	0.522	0.371	1.943	1.139	0.092
CCL17, pg/mL	-0.001	0.009	0.883	-0.017	0.028	0.542	-0.354	0.375	0.347	2.166	0.992	0.032
Cytokines in sputum supernatant												
IL-1β, pg/mL	-0.218	0.028	<0.001	0.382	0.052	<0.001	-0.298	0.418	0.478	1.631	0.779	0.039
TNF-α, pg/mL	-0.164	0.048	0.003	0.136	0.070	0.053	-0.487	0.423	0.253	1.748	0.833	0.038
IFN-γ, pg/mL	-1.167	0.780	0.138	-3.155	1.815	0.085	-0.481	0.420	0.256	1.946	0.836	0.289
IL-5, pg/mL	0.049	0.237	0.837	-0.323	0.408	0.430	-0.522	0.425	0.222	1.835	0.844	0.032
IL-6, pg/mL	-0.017	0.011	0.121	-0.001	0.026	0.966	-0.573	0.420	0.176	1.852	0.847	0.031
IL-8, pg/mL	0.000	0.001	0.725	0.001	0.001	0.438	-0.526	0.425	0.219	1.845	0.843	0.031
CCL22, pg/mL	-0.002	0.013	0.898	0.001	0.031	0.967	-0.529	0.432	0.222	1.862	0.872	0.035

FEV_1: forced expiratory volume in 1 second; Δ: change from the baseline.

*: All models were adjusted for pre-FEV_1, % predicted; gender; body mass index; smoking; asthma duration; inhaled corticosteroid daily dosage; and anxiety symptom scores of the Hospital Anxiety and Depression Scale.

#: All models were adjusted for age, gender, body mass index, smoking, asthma duration, inhaled corticosteroid dosage and anxiety symptom of the Hospital Anxiety and Depression Scale.

951 **Figure legend**



952

953 Figure E1. Baron and Kenny mediational model of the conceptual relationship among
954 depressive symptoms, inflammatory mediator and bronchodilator response (BDR).
955 Values in parentheses are standard errors of those path coefficients; β_1=raw
956 (unstandardized) regression coefficient for the association between depressive
957 symptoms and inflammatory mediator; SE_1=standard error of β_1; β_2 =raw
958 (unstandardized) regression coefficient for the association between depressive
959 symptoms and BDR in the absence of inflammatory mediators; SE_2=standard error of
960 β_2; β_3=raw (unstandardized) regression coefficient for the association between
961 inflammatory mediator and BDR in the absence of depressive symptoms; SE_3=standard
962 error of β_3; β'_2=raw coefficient for the association between depressive symptoms and
963 BDR when an inflammatory mediator was also a predictor of the BDR; SE'_2=standard
964 error of β'_2; β'_3=raw coefficient for the association between the inflammatory mediator
965 and the BDR when the depressive symptoms were also a predictor of the BDR;

966 SE'_3=standard error of β'_3.

967