Exacerbating Factors Induce Different Gene Expression Profiles in Peripheral Blood Mononuclear Cells from Asthmatics, Patients with Chronic Obstructive Pulmonary Disease and Healthy Subjects

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Key Words

Airway inflammation · Asthma · Chronic obstructive pulmonary disease · Exacerbating factors · House dust mites · Lipopolysaccharides · TaqMan low density array

Abstract

Background: Despite several common phenotypic features, chronic obstructive pulmonary disease (COPD) and severe asthma differ with regard to their causative factors and pathophysiology. Both diseases may be exacerbated by environmental factors, however, the molecular profiles of disease episodes have not been comprehensively studied. We identified differences in gene and protein expression profiles expressed by peripheral blood mononuclear cells (PBMC) of COPD patients, patients with atopic asthma and healthy subjects when challenged with exacerbating factors in vitro: lipopolysaccharide (LPS), house dust mite (HDM) and cat allergen. **Methods:** PBMC isolated from patients with severe atopic asthma and COPD, as well as healthy subjects were stimulated with rDer p 1 DG, rFel d 1 DG and LPS. The changes in the expression of 47 genes belonging to five groups (phospholipase A₂, eicosanoids, transcription factors, cytokines and airway remodeling) were studied using

TaqMan low density array cards. Immunoblotting was used to study relative protein expression. **Results:** rDer p 1 significantly up-regulated the expression of *PLA2G4A*, *PLA2G6*, *PLA2G15*, *CYSLTR1*, *LB4R2*, *PTGS1*, *PTGS2*, *FOXP1*, *GATA3*, *HDAC2*, *IREB2*, *PPARG*, *STAT4*, *TSLP* and *CHI3L1* genes in asthmatics in comparison to healthy subjects. LPS induced significant expression of *ANXA1* and *LTA4H* in asthmatics when compared to COPD patients and healthy subjects. *SOX6*, *STAT4* and *IL1RL1* were induced in COPD after LPS stimulation. Analysis of protein expression revealed a pattern similar to mRNA expression. **Conclusions:** LPS-induced exacerbation of asthma and COPD is characterized by differential expression of selected genes in PBMC. HDM allergen changed the expression profile of inflammatory genes between patients with asthma of atopic origin and healthy controls.

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Introduction

Both asthma and chronic obstructive pulmonary disease (COPD) are airway diseases with a number of common phenotypic features, such as chronic airway inflammation and impaired airflow. However, important differ-

ences exist between asthma and COPD pathogenesis. In total, 70% of asthma cases have an atopic origin, whereas COPD is usually associated with cigarette smoking and exposure to noxious agents. In addition, the distribution of causative agents in the respiratory tract influence disease localization: while asthma affects the large and small airways, COPD impairs the function of small airways [1]. Furthermore, while asthma starts early in life, COPD is usually diagnosed in patients older than 45 years. Mast cells, Th CD4+ cells and eosinophils play a pivotal role in asthma, and neutrophils, Th CD8+ and macrophages characterize COPD inflammation. Despite these differences, acute exacerbation presents in both diseases and sometimes requires hospitalization. However, it is not clear whether exacerbating factors initiate the same cascade of signaling pathways in both diseases.

Causative factors, either allergens or viral/bacterial infections, are known to lead to inflammation enhancement. Airway inflammation is characterized by the presence of many inflammatory mediators, among which lipid derivatives have been described in detail. Leukotrienes and prostaglandins are responsible for airway muscle contraction, wheezing and mucus secretion. Their production is initiated by the release of arachidonic acid through the action of phospholipase A₂. Our previous studies have shown that cytosolic phospholipase A₂ is involved in the pathogenesis of asthma [2, 3]. Moreover, the involvement of different phospholipases in asthma and COPD pathogenesis has been stated recently [4]. The cells that are recruited in large number to the airways as well as those in the peripheral blood start to produce cytokines in response to trigger factors [5–10]. Activation of transcription factors, such as *SOX6*, *FOXP1*, *GATA3*, *HDAC2*, *STAT4*, *IREB2* and *PPARG*, influence the course of inflammation. Through binding to the CtBP2 co-repressor and HDAC1-histone deacetylase, *SOX6* is able to induce transrepression. Moreover, it might block canonical Wnt signaling in T cells [11, 12] and facilitates cardiac and skeletal muscle differentiation [13]. Bhattacharya et al. [14] demonstrated that *SOX6* expression in peripheral blood mononuclear cells (PBMC) of COPD patients positively correlated with lung function. *FOXP1* is a transcription factor involved in lung development, cell proliferation and differentiation, as well as signal transduction [15, 16]. It regulates monocyte differentiation and macrophage function [17] and through interaction with HDAC₂, it can influence IL-6 expression [18]. *GATA3* is a transcription factor involved in T-cell development and promotes the secretion of IL-4, IL-5 and IL-13. Genome-wide association studies identified an association be-

tween *IRBE2*, one of the genes located in chromosome region 15q25.1, and COPD as well as airway obstruction [19].

Persistent airway inflammation finally results in airway remodeling and lack of airway elasticity. Metalloproteinases and chitinases participate in this process [20, 21]. Matrix metalloproteinase (MMP)-9 has been shown to increase during COPD exacerbation, and its level has been correlated with neutrophil and lymphocyte counts in sputum [22]. The *CHI3L1* gene encodes YKL-40, a chitinase-like protein also known as human cartilage glycoprotein-39. Although chitinase-like proteins differ from chitinases in so far that the former binds to chitin but does not hydrolyze it, while the latter possesses catalytic properties, both enzymes are able to modulate immune responses. YKL-40 acts as a soluble mediator of cell proliferation and migration [23, 24]. Its level correlates with asthma severity and airway remodeling measured as the thickness of the subepithelial basement membrane [25]. Moreover, polymorphisms in *CHI3L1* are associated with the risk of atopy [26, 27]. However, comprehensive data elucidating the molecular changes induced by exacerbating factors in both COPD and asthma more clearly are still lacking.

The present study uses PBMC as an experimental model for two reasons. Changes in PBMC are easily observable, and they can be easily employed for possible diagnostic approaches. The presence of systemic inflammation in chronic diseases also influences the function of PBMC: T lymphocytes have been reported to have impaired function in COPD, and the monocytes of COPD patients produce more MMP-9 and IL-6, and show NF- κ B activation [28]. The existence of systemic inflammation in COPD is associated with the presence of systemic COPD comorbidities, including chronic heart failure, diabetes and arteriosclerosis [29]. Systemic inflammation in asthma is less prominent, but increased levels of peripheral eosinophils has been found to correlate with disease severity and lung function [30]. Patients with allergic rhinitis and asthma have a higher percentage of Th17 lymphocytes than healthy controls, and exposure to allergen further increases their number [31, 32]. In addition, total leukocyte counts and blood markers such as C-reactive protein are elevated in asthmatics [33]. Most blood monocytes of asthmatics are known to be CD14+/CD16+ and show features of tissue macrophages, such as enhanced release of superoxide anions, and glucocorticoids may influence the numbers of CD14+/CD16+ and CD14+/CD16+ monocytes in asthma patients [34, 35]. Monocytes and CD4+ T cells

from patients with allergic rhinitis express a number of phenotypic alternations [36].

The aim of the present study was to determine how PBMC of asthmatics and COPD patients respond to key exacerbating factors: allergen in asthma and LPS in asthma and COPD.

Materials and Methods

Reagents

LoTox™ deglycosylated recombinant *Dermatophagoides pteronyssinus* allergen 1 (rDer p 1 DG) and LoTox deglycosylated recombinant *Felis domesticus* allergen 1 (rFel d 1 DG) were purchased from Indoor Biotechnologies (Cardiff, UK). LPS from *Escherichia coli* serotype R515 was obtained from Enzo Life Sciences (New York, N.Y., USA). TaqMan low density array (TLDA) cards and a high-capacity cDNA kit were ordered from Life Technologies (Carlsbad, Calif., USA). Histopaque 1077, RPMI 1640, FBS, penicillin, streptomycin, RIPA buffer and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Polymyxin B and BCIP/NBT alkaline phosphatase substrate were purchased from Merck Millipore (Darmstadt, Germany). The RNeasy cell mini kit with QIAshredder and DNase set were purchased from Qiagen (Hilden, Germany). The BCA protein assay kit was ordered from Pierce Thermo Scientific (Rockford, Ill., USA). The following antibodies were used: annexin-1 and β -actin from Cell Signaling (Danvers, Mass., USA); iPLA₂, LTA₄H and COX-2 from Cayman Chemical (Ann Arbor, Mich., USA); HDAC₂ and PPAR- γ from Santa Cruz Biotechnology (Dallas, Tex., USA), and PLA₂XV and YKL-40 from Abcam (Cambridge, Mass., USA).

Patients

Patients with severe asthma of atopic origin or COPD, as well as healthy volunteers, were enrolled in the study. The project was approved by the local bioethical committee and informed consent was obtained from all the subjects. The subjects were recruited from the Department of Internal Diseases, Asthma and Allergy of the Medical University of Lodz. Asthma had to be diagnosed at least 6 months prior to the study and meet the criteria of the Global Initiative for Asthma [37]. The severity of the disease was assessed according to the American Thoracic Society Workshop on Refractory Asthma 2000 report [38]. COPD patients were enrolled in the study if they met the following criteria: (a) presence of clinical symptoms of chronic airway inflammation confirmed by persistent cough with sputum production lasting more than 3 months/year during the last 2 years, (b) FEV₁ <80% and FEV₁/FVC ratio <70%, (c) a negative bronchodilator test, and (d) lack of other dysfunctions in the respiratory tract. Nonatopic subjects with a negative family history of allergy and asthma were included as healthy controls.

Patients were asked to abstain from antihistamine drugs, oral glucocorticoids or leukotriene receptor antagonists for 24 h, and glucocorticoid inhalation or long-acting β -agonist use 12 h before blood was drawn.

Detailed characteristics of the patients and controls are shown in table 1.

Table 1. Parameters and characteristics of severe asthmatics, COPD patients and controls

Parameter	Asthmatics	COPD	Healthy controls
n	11	6	7
Males/females	2/9	3/3	1/6
Race	Caucasian	Caucasian	Caucasian
Age, years	48	63	41
Range	36–69	53–71	29–56
FEV ₁	75.9	47.8	N/A
Range	55–94	41–55	–
FEV ₁ /FVC	68.7	50.8	N/A
Range	56.8–86.1	40.1–58.6	–
PEF, liters	5.8	3.85	N/A
Range	3.3–9.3	2.7–6.5	–
Allergic to HDM	10	0	0
Allergic to cat dander	8	0	0
Current/nonsmokers	0/0	1/5	0/0

Data are presented as means and ranges. PEF = Peak expiratory flow.

Methods

PBMC Isolation and Stimulation. PBMC were isolated by centrifugation on Histopaque 1077, a density gradient cell separation medium, according to the manufacturer's instructions. Cells were then cultured in RPMI 1640 with 10% heat-inactivated FBS, 2 mM L-glutamine and 10 ng/ml polymyxin B (allergens) or 100 U/ml penicillin and 100 μ g/ml streptomycin. 2×10^6 /ml PBMC were stimulated in vitro with LoTox deglycosylated rDer p 1 and LoTox deglycosylated rFel d 1 for 6 days or LPS from *E. coli* serotype R515 for 8 h.

RNA Extraction and cDNA Synthesis. Total RNA was isolated from PBMC using the RNeasy cell mini kit with QIAshredder according to the manufacturer's instructions. RNA was DNase treated, purified, eluted in 30 μ l of RNase-free water and stored at -80°C for further analysis. RNA was then reverse transcribed to cDNA using a high-capacity cDNA kit.

TLDA Cards. Each card of the low-density array contains eight separate loading ports that feed into 48 separate wells for a total of 384 wells per card. Each 2- μ l well contains a lyophilized TaqMan assay to enable a single gene to be detected. In this study, the TLDA card was configured into eight identical 48-gene sets (table 2). Each set of 48 genes also contains two reference genes: *RNASEP1* and *GAPDH*. The coefficient of variation of Cq (quantification cycle) for the *GAPDH* reference gene between compared arrays was less than 5%.

Real-Time PCR. 400 ng of cDNA were used for each port of TLDA. The array was centrifuged twice for 1 min each at 306 g to distribute the samples from the loading port into each well. The card was then sealed and quantitative PCR (qPCR) was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems). RQ Manager 1.2.1 software was used to analyze raw qPCR data. The results were analyzed in comparison to the expression of the reference gene, using Livak's method, and presented as the relative expression of mRNA in the form of $\text{RQ} = 2^{-\Delta\Delta\text{Cq}}$. The

Table 2. Gene-specific results of allergen and LPS stimulation

Gene symbol	Gene name
<i>Gene-specific results of rDer p 1 stimulation</i>	
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium dependent)
PLA2G6	phospholipase A2, group VI (cytosolic, calcium independent)
PLA2G15	phospholipase A2, group XV
PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
CYSLTR1	cysteinyl leukotriene receptor 1
LTB4R	leukotriene B4 receptor
FOXP1	forkheadbox P1
GATA3	GATA binding protein 3
HDAC2	histone deacetylase 2
IREB2	iron-responsive element binding protein 2
PPARG	peroxisome proliferator-activated receptor- γ
STAT4	signal transducer and activator of transcription 4
TSLP	thymic stromal lymphopoietin
CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
<i>Gene-specific results of LPS stimulation</i>	
ANXA1	annexin A1
LTA4H	leukotriene A4 hydrolase
IL1RL1	interleukin 1 receptor-like 1
SOX6	SRY (sex-determining region Y)-box 6
STAT4	signal transducer and activator of transcription 4

untreated sample was used as a calibrator. The $\log_2$ base transformation of the RQ value was used to show changes in gene expression (i.e. RQ = 10; $\log_2 10 = 3.332$).

Immunoblotting. Total protein from PBMC of patients with asthma and COPD and healthy controls was extracted in RIPA protein extraction buffer supplemented with protease inhibitor. Protein concentrations were determined by the BCA protein assay kit; 20 μg of total protein were subjected to electrophoresis in 4–12% gels at 140 V and transferred to a nitrocellulose membrane using the eBlot Protein Transfer System. The membrane was blocked with 5% nonfat milk in TBST for 1 h at room temperature. Then, membranes were incubated for 12 h at 4°C with the primary antibodies and subsequently for 1 h with secondary antibodies at room temperature. The band was developed using BCIP/NBT alkaline phosphatase substrate. Densitometric analysis of bands was performed with ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, Bethesda, Md., USA) and the results are presented as fold change in optical density.

Statistical Analyses

Data were analyzed using Statistica software (v.10.0; StatSoft, Tulsa, Okla., USA). The distribution of the $\log_2$ data and the equality of variances were checked by Shapiro-Wilk and Levene's tests, respectively. Significant changes were determined by ANOVA with the Tukey post hoc test as multiple comparison procedure.

The same procedure was used for each comparison (severe asthma vs. healthy subjects and severe asthma vs. COPD vs. healthy subjects). Values of $p < 0.05$ were considered statistically significant.

Results

Gene Expression

D. pteronyssinus Allergen 1

Sensitivity to house dust mites (HDM) is a risk factor associated with the development of asthma [39]. Further exposure to allergen can cause symptom exacerbation, which increases patient suffering and requires additional medical expenditure. As the asthmatics who participated in our study were atopic to Der p 1, PBMC isolated from the blood of those patients were stimulated in vitro with rDer p 1 allergen (1 $\mu\text{g}/\text{ml}$). qPCR was then used to study the expression of 47 genes involved in the pathophysiology of chronic airway inflammation.

Phospholipases A₂. The present study examined the expression of eight phospholipase A₂, phospholipase A₂ receptor (PLA2R1) and phospholipase A₂ inhibitor (ANXA1) genes. A 6-day stimulation with rDer p 1 caused significant up-regulation of PLA2G4A (asthmatics, A: 1.96 ± 0.49 vs. healthy controls, H: 0.27 ± 0.36 ; $p = 0.018$), PLA2G15 (A: 1.65 ± 0.41 vs. H: 0.2 ± 0.29 ; $p = 0.02$) and PLA2G6 (A: 1.51 ± 0.41 vs. H: 0.32 ± 0.21 ; $p = 0.016$) genes in asthmatics (fig. 1). Genes of low-molecular-weight secretory PLA₂ (GIIA, V and X) were hardly detectable in the participants. Expression of PAFAH1B and ANXA1 genes tended to be up-regulated in healthy controls in comparison to asthmatics, whereas a trend to increased PLA2G7 and PLA2R1 expression was observed in asthmatics.

Arachidonic Acid. Among the genes belonging to the eicosanoid pathway, four demonstrated significantly altered expression as a result of rDer p 1 stimulation. Two genes of leukotriene receptors, CYSLTR1 (A: 1.28 ± 0.32 vs. H: 0.42 ± 0.26 ; $p = 0.005$) and LTB4R2 (A: 1.03 ± 0.31 vs. H: 0.14 ± 0.27 ; $p = 0.035$), were up-regulated in both groups, but expression was higher in asthmatic patients. Also, expression of genes for COX-1 (PTGS1, A: 1.55 ± 0.44 vs. H: 0.14 ± 0.28 ; $p = 0.04$) and COX-2 (PTGS2, A: 1.76 ± 0.62 vs. H: -0.05 ± 0.48 ; $p = 0.04$) was increased in asthmatics (fig. 2).

Transcription Factors. A significant difference in the expression of six transcription factor genes was observed between asthmatics and controls (FOXP1, A: 1.29 ± 0.41 vs. H: 0.23 ± 0.24 , $p = 0.02$; GATA3, A: 1.24 ± 0.41 vs. H: 0.22 ± 0.22 , $p = 0.04$; HDAC2, A: 1.74 ± 0.38 vs. H: $0.5 \pm$

Fig. 1. Expression of phospholipase A₂ genes in PBMC of healthy controls (n = 5) and patients with severe asthma (n = 5) after stimulation with rDer p 1 DG (1 µg/ml). Means of log₂ RQ ± SE, * p < 0.05.

Fig. 2. Expression of eicosanoid pathway genes in PBMC of healthy controls (n = 5) and patients with severe asthma (n = 5) after stimulation with rDer p 1 DG (1 µg/ml). Means of log₂ RQ ± SE, * p < 0.05.

0.41, $p = 0.04$; *IREB2*, A: 1.3 ± 0.36 vs. H: 0.35 ± 0.16 , $p = 0.008$; *PPARG*, A: 2.22 ± 0.66 vs. H: 0.43 ± 0.26 , $p = 0.014$; *STAT4*, A: 1.18 ± 0.41 vs. H: 0.29 ± 0.17 , $p = 0.04$; fig. 3). The *PPARG* gene was the highest expressed transcription factor gene in asthmatics.

Cytokines and Remodeling. PBMC of asthmatic patients showed increased expression of *TSLP* (A: 1.59 ± 0.39 vs. H: 0.25 ± 0.31 ; $p = 0.02$) and *CHI3L1* (A: 2.29 ± 0.73 vs. H: 0.39 ± 0.27 ; $p = 0.01$) genes after stimulation with rDer p 1 (fig. 4). The *IL33* gene was barely detected in both groups. All other cytokine genes show a tendency to a higher expression in asthmatics than controls.

Lipopolysaccharides

At least 50% of COPD patients have bacteria in the lower airways during disease exacerbation, and, in most of them, the bacteria remain in the respiratory tract in the stable phase of COPD [40–42]. Bacteria may also worsen the course of asthma [43]. In the present study, PBMC from COPD patients, asthmatics and healthy subjects

were stimulated with LPS (100 ng/ml) for 8 h, and the differences in the expression of the studied genes were checked between groups.

Phospholipase A₂. PBMC stimulation with LPS resulted in significantly different expression of *ANXA1* between asthmatics (0.36 ± 0.29), COPD patients (-0.64 ± 0.21) and controls (-0.56 ± 0.19 ; $p = 0.04$; fig. 5). Although not significant, the profiles of *PLA2G15* and *PLA2R1* expression were similar to that of *ANXA1*. A trend to increased expression of *PLA2G4* and *PLA2G7* was noted in all study groups. Genes for *PLA2G2*, *PLA2G5* and *PLA2G10* were hardly detected. Only the *PAFAH1B* gene showed a tendency to be increased in healthy controls and COPD patients and down-regulated in asthmatics.

Arachidonic Acid. *LTA4H* was up-regulated in asthmatics (0.5 ± 0.38) and down-regulated in both COPD patients (-0.87 ± 0.27 ; $p = 0.01$) and healthy volunteers (-0.74 ± 0.25 ; $p = 0.03$; fig. 6). Of all the eicosanoid pathway genes, *CYSLTR1*, *CYSLTR2* and *LTB4R2* showed a trend to up-regulation in COPD and down-regulation in

Fig. 3. Expression of transcription factor genes in PBMC of healthy controls (n = 5) and patients with severe asthma (n = 5) after stimulation with rDer p 1 DG (1 µg/ml). Means of $\log_2$ RQ $\pm$ SE, * $p < 0.05$.

Fig. 4. Expression of cytokine genes and genes related to airway remodeling in PBMC of healthy subjects (n = 5) and patients with severe asthma (n = 5) after stimulation with rDer p 1 DG (1 µg/ml). Means of $\log_2$ RQ $\pm$ SE, * $p < 0.05$.

healthy controls and asthmatics. While not significant, *PTGS2* and *LTC4S* genes exhibited a positive expression shift in all three groups, whereas *PTGS1* expression was increased only in asthmatics. *ALOX12* was found to have a tendency to be negatively regulated in asthmatics after LPS stimulation

Transcription Factors. LPS significantly changed the expression of *SOX6* and *STAT4* only in COPD patients (*SOX6*, 0.64 ± 0.23 ; *STAT4*, 0.77 ± 0.35) compared with the control group (*SOX6*, -0.27 ± 0.23 , $p = 0.008$; *STAT4*, 0.27 ± 0.12 , $p = 0.04$; fig. 7). Although not significant, only *PPARG* gene expression was increased in asthmatics

and down-regulated in COPD patients and healthy subjects.

Cytokines and Remodeling. After stimulation with LPS, PBMC from COPD patients (1.47 ± 0.37) displayed elevated expression of *IL1RL1* in comparison to asthmatics (0.72 ± 0.3 ; $p = 0.02$) and healthy subjects (0.95 ± 0.22 ; $p = 0.04$; fig. 8). The expression of all other cytokines showed a tendency to be increased after LPS treatment, with the highest RQ values being noticed for *IL8* and *IL1B*. We did not observe significant changes in the expression of *CHI3L1* and *MMP9* in LPS-stimulated PBMC between asthmatics, COPD patients and healthy subjects.

Fig. 5. Expression of the *ANXA1* gene in PBMC of healthy controls (n = 7) and patients with severe asthma (n = 5) and COPD (n = 6) after stimulation with LPS (100 ng/ml). Means of $\log_2$ RQ $\pm$ SE, * $p < 0.05$.

Fig. 6. Expression of the *LTA4H* gene in PBMC of healthy controls (n = 7) and patients with severe asthma (n = 5) and COPD (n = 6) after stimulation with LPS (100 ng/ml). Means of $\log_2$ RQ $\pm$ SE, * $p < 0.05$.

Fig. 7. Expression of *SOX6* and *STAT4* genes in PBMC of healthy controls (n = 7) and patients with severe asthma (n = 5) and COPD (n = 6) after stimulation with LPS (100 ng/ml). Means of $\log_2$ RQ $\pm$ SE, * $p < 0.05$.

Protein Expression

Based on data from qPCR, we assessed the relative protein expression of cPLA $_2\alpha$, iPLA $_2$, PLA $_2$ XV, COX-2, HDAC $_2$, PPAR- γ , YKL-40 in PBMC of asthmatics and healthy subjects stimulated with rDer p 1. The relative expression of annexin 1 and LTA $_4$ H was measured in PBMC stimulated with LPS.

Phospholipases A $_2$. PBMC from asthmatics showed an increase in the relative protein expression of PLA $_2$ XV in comparison to healthy subjects (A: 0.14 ± 0.06 ; H: -0.19 ± 0.09). While not significant, the relative protein expression of iPLA $_2$ and cPLA $_2\alpha$ tended to be ele-

vated in asthmatics (fig. 9). The relative protein expression of annexin-1 was increased in freshly isolated, untreated PBMC from COPD patients when compared with asthmatics and healthy subjects (H: 0.26 ± 0.01 vs. A: 0.29 ± 0.01 vs. COPD: 0.44 ± 0.8). LPS caused a decrease in relative protein expression of annexin-1 in patients with COPD (H: 0.13 ± 0.16 vs. COPD: -0.3 ± 0.22 ; fig. 10)

Transcription Factors. The relative protein expression of HDAC $_2$ and PPAR- γ was assessed in PBMC stimulated with rDer p 1. Asthmatics showed decreased expression of HDAC $_2$ (H: 0.11 ± 0.05 vs. A: -0.29 ± 0.16 ; fig. 11).

Fig. 8. Expression of the *IL1RL1* gene in PBMC of healthy controls (n = 7) and patients with severe asthma (n = 5) and COPD (n = 6) after stimulation with LPS (100 ng/ml). Means of log₂ RQ ± SE, * p < 0.05.

Fig. 9. Relative PLA₂XV, cPLA₂α and iPLA₂ protein expression in PBMC from healthy controls (H) and asthmatic patients (A) in vitro stimulated with rDer p 1. PBMC (2×10^6) were stimulated with 1 μg/ml rDer p 1 for 6 days. Immunoblots are representative of experiments in PBMC from 6 donors each showing similar re-

sults. The box plots show densitometric results. Data are presented as the fold change compared with the vehicle-treated cells (control) both normalized to β-actin. Means ± SE, * p < 0.05. A1C, A2C, A3C, H1C, H2C = PBMC treated with medium only.

There were no differences in relative PPAR-γ expression between the study groups.

Eicosanoid Pathway. The relative COX-2 expression was assessed in rDer p 1-stimulated PBMC from asthmatics and healthy controls as well as relative protein expression of LTA₄H in LPS-stimulated PBMC from asthmatics, controls and COPD patients. Although not

significant, both proteins had relative expression profiles similar to those introduced for mRNA expression (fig. 12).

Cytokines and Remodeling. Stimulation with rDer p 1 results in a significant difference in relative YKL-40 expression in PBMC between healthy controls (-0.72 ± 0.18) and asthmatics (0.12 ± 0.07 ; fig. 13).

Fig. 10. Relative annexin-1 protein expression in PBMC from healthy controls (H) and patients with asthma (A) and COPD (C) at baseline (**a**) and after in vitro stimulation with LPS (**b**). **a** Freshly isolated PBMC without culturing. **b** PBMC (2×10^6) stimulated with 100 ng/ml LPS for 8 h. Immunoblots are representative of

experiments in PBMC from 6 donors each showing similar results. The box plots show densitometry results. **a** Data normalized to β-actin. **b** Data compared with the vehicle-treated cells (control) both normalized to β-actin. Means $\pm$ SE, * $p < 0.05$. A1C, A2C, H1C, H2C, C1C = PBMC treated with medium only.

Fig. 11. Relative HDAC₂ (**a**) and PPAR-γ (**b**) protein expression in PBMC from healthy controls (H) and asthmatics (A) stimulated in vitro with rDer p 1. PBMC (2×10^6) were stimulated with 1 μg/ml rDer p 1 for 6 days. Immunoblots are representative of experiments in PBMC from 6 donors each showing similar results. The

box plots show densitometry results. Data are presented as the fold change compared with the vehicle-treated cells (control) both normalized to β-actin. Means $\pm$ SE, * $p < 0.05$. A1C, A2C, H1C, H2C = PBMC treated with medium only.

Discussion

Exacerbations in asthma and COPD are characterized by an enhanced inflammatory response, intensive recruitment of inflammatory cells and a higher concentration of inflammatory mediators. This explorative investigation assesses the response of PBMC isolated from atop-

ic asthmatics, COPD patients and healthy subjects to three exacerbating factors: indoor HDM allergen (Der p 1), cat dander allergen (Fel d 1) and bacterial factor (LPS). As the COPD patients were not atopic and allergens are etiological factors of asthma, only PBMC of asthmatics were stimulated with allergens. However, as bacterial infection is known to worsen the course of both diseases,

Fig. 12. Relative LTA₄H expression in PBMC from healthy controls (H) and patients with asthma (A) and COPD (C) stimulated with LPS (**a**) and relative COX-2 expression in PBMC stimulated in vitro with rDer p 1 (**b**). PBMC (2×10^6) were stimulated with 1 μ g/ml rDer p 1 for 6 days or 100 ng/ml LPS for 8 h. Immunoblots are representative of experiments in PBMC from 6 donors each

showing similar results. The box plots show densitometry results. **a** Data normalized to β -actin. **b** Data are presented as the fold change compared with the vehicle-treated cells (control) both normalized to β -actin. Means $\pm$ SE, * $p < 0.05$. A1C, A2C, H1C, H2C, C1C, C2C = PBMC treated with medium only.

Fig. 13. Relative YKL-40 protein expression in PBMC from healthy controls (H) and patients with asthma (A) and COPD (C) at baseline (**a**) and after in vitro stimulation with rDer p 1 (**b**). **a** Freshly isolated PBMC without culturing. **b** PBMC (2×10^6) stimulated with 1 μ g/ml rDer p 1 for 6 days. Immunoblots are representative

of experiments in PBMC from 6 donors each showing similar results. The box graph shows densitometry results. **a** Data normalized to β -actin. **b** Data presented as fold change compared with vehicle-treated cells (control) both normalized to β -actin. Means $\pm$ SE, * $p < 0.05$. A1C, A2C, H1C, H2C = PBMC treated with medium only.

LPS was used to stimulate PBMC from both patient groups. We used recombinant allergens as the extracts may give false results [44]. The aim of the study was to determine, firstly, whether in vitro exposure to allergens differs between allergic asthmatics and healthy controls

in relation to the involvement of selected phospholipase A₂ enzymes, eicosanoid pathway genes, cytokines and transcription factors; secondly, whether LPS modulates the expression of these genes similarly in patients with COPD and those with asthma, and finally, which of the

tested agents has the greatest impact on the PBMC transcription profile in the groups studied.

The first group of genes analyzed comprised phospholipase A₂ genes, PLA₂ receptor and PLA₂ inhibitor. rDer p 1 was found to be the most potent inducer of their expression among the agents tested, and asthmatics were the group showing significant changes in their expression. The results from qPCR were used to study the relative protein expression of selected genes. The present study is the first to show that *PLA2G15* can be regulated by Der p 1 in the PBMC of asthmatics. *PLA2G15*, a relatively newly identified, 45-kDa glycoprotein, was first discovered in 1996 [45] in canine MDCK cells and later in humans, but its biological function remains to be fully elucidated. It possesses PLA₂ and PLA₁ activity insofar as it can hydrolyze the acyl chain at the sn-1 and sn-2 positions of phosphatidylcholine and phosphatidylethanolamine, and is localized in lysosomes. It is Ca²⁺ independent but shows increased activity in environments with low concentrations of Ca²⁺ or Mg²⁺. Currently, PLA₂G15 is considered to be primarily an enzyme involved in lipid metabolism in alveolar macrophages and is believed to be associated with phospholipidosis. Further information related to the biological function of PLA₂G15 has been obtained from recent studies on its role in iNKT cell-mediated CD1d recognition. The normal selection and maturation of iNKT cells requires complexes of CD1d with thymic lipids, whose generation is PLA₂G15 dependent [46], which is particularly pertinent in this context, as iNKT cells can be activated by HDM extracts [47].

The data regarding transcriptional control of the *PLA2G15* gene is limited. It has been established that retinoic acid stimulates *PLA2G15* gene activation via an RXR-dependent pathway, but this effect was observed only in THP-1 cells, not in COS-7, MDCK or T293 cells [48]. The mechanism by which Der p 1 exerts an impact on *PLA2G15* expression remains to be established. Der p 1 can act by both the PAR-2 receptor as well as in a PAR-2-independent manner by activation of NF-κB and ERK1/2 [49, 50]. As the *PLA2G15* gene has binding sites for NF-κB as well as for ATF-2, ELK-1, SP2-1 (Sabiosciences) and ERK1/2-regulated transcription factors, the involvement of the Der p 1-NF-κB pathway should be verified.

PBMC of asthmatics stimulated with rDer p 1 showed increased expression of *PLA2G4A* in comparison to healthy controls. These results confirm those of a previous study where eosinophils isolated from asthmatics and challenged with Der p 1 and Fel d 1 showed increased ac-

tivity and cPLA₂ phosphorylation [51]. In addition, Whalen et al. [52] demonstrated that allergen-induced (Der p 1, Fel d 1 and ragweed) cytokine expression in PBMC is attenuated after inhibition of cPLA₂α. These observations suggest that allergens may influence the expression or activity of cPLA₂α. As cPLA₂ and iPLA₂ (*PLA2G6*) also participate in leukocyte chemotaxis [53], increased expression of these proteins may also have an impact on further translocation of these cells. Although the differences in relative protein expression of cPLA₂ and iPLA₂ between asthmatics and controls were not significant, it was noticeable that the protein expression profile tended to be similar to the mRNA profile.

ANXA1, also known as lipocortin 1, a PLA₂ inhibitor, was significantly changed in asthmatics after LPS stimulation. Inflammation is a self-resolving process and this can be achieved by activation of some anti-inflammatory genes such as *ANXA-1*. It has been demonstrated that a 6-hour stimulation with LPS results in increased *ANXA* expression in murine PBMC, which returns to the baseline value 24 h after stimulation [54]. This observed significant change can be a characteristic feature for the time point of stimulation chosen and not for long LPS action. Analysis of relative protein expression showed that COPD patients had significantly higher relative *Anxa-1* expression than controls, however, LPS stimulation resulted in decreased *Anxa-1* concentrations. The regulation of gene expression includes many steps from mRNA synthesis to protein translation: processing of mRNA, transport of mRNA to the cytoplasm, translation and posttranslational modifications. All of these processes may be the potential cause of the observed differences between mRNA and protein expression.

The second group contained genes connected with the eicosanoid pathway. rDer p 1 was found to significantly change the expression of *PTGS1* and *PTGS2* genes, and in this regard our results contradict those of Sánchez-Borges et al. [55] where HDM extract inhibited COX-1. The authors reported that this effect can be caused by COX-1-inhibitory substances in commercial mite allergenic extracts rather the allergen alone; the lack of any inhibitory effect observed in the present study can be explained by our use of the recombinant allergen (rDer p 1). On the other hand, Herreras et al. [56] observed in a mouse model that HDM-induced airway hyperreactivity and inflammation were associated with increased COX2 mRNA production. In our experiments, Der p 1 also induced the expression of *LTB4R* and *CYSLTR1* genes. HDM allergens are able to stimulate the robust production of cysteinyl leukotriene from mouse bone marrow-derived dendritic cells [57] as well as to up-regulate the expression of

CYSLTR1 on these cells [58]. These observations may suggest that eicosanoids are involved in the Der p 1-induced increase in inflammatory processes in asthma.

The third group consisted of cytokine genes. In our study, Der p 1 significantly increased the expression of the *TSLP* gene. Thymic stromal lymphopoietin (TSLP) was reported to be produced by epithelial cells and keratinocytes, and known to influence the function of dendritic cells, which can further activate T cells. Only one study confirmed that human monocytes and monocyte-derived dendritic cells show TSLP gene expression, which can be also induced by LPS or zymosan [59]. Kashyap et al. [59] also noted that murine lung epithelial cells and dendritic cells produce TSLP in response to HDM extract. The ability of Der p 1 to induce TSLP secretion by BEAS-2B, a human bronchial epithelial cell line, was further confirmed by Al-Ghoul et al. [60].

LPS was found to selectively and significantly up-regulate the expression of *IL1RL1* (ST2) in COPD patients when compared with other groups. A recent study using a mouse model showed that the expression levels of IL-33 and ST2 were markedly enhanced in the lung tissue after inhalation of cigarette smoke [61]. This observation supports those presented by Hacker et al. [62], who reported increased serum levels of soluble ST-2 in COPD patients compared with healthy controls.

The fourth set comprised transcription factor genes, e.g. *SOX6* and *STAT4*, which were significantly induced by LPS in COPD patients, and *FOXP1*, *GATA3*, *HDAC2*, *STAT4*, *IREB2* and *PPARG* were up-regulated by rDer p 1 allergen. In a mouse model, the *STAT4* pathway was linked to allergen-induced chemokine production and airway hyperreactivity [63]. *STAT4* is involved in IFN- β -induced MCP-1 mRNA expression in mast cells [64]. As both LPS and Der p 1 were found to induce expression of the *STAT4* gene, this pathway may be involved in LPS- and allergen-induced airflow impairment during exacerbations. Macaubas et al. [65] reported that *GATA3* expression is induced by HDM extract in the PBMC of atopic volunteers. As the authors suggested, monitoring of transcription factor expression can be used to control Th2 responses, and our study shows that this approach can also be used in patients with asthma of atopic origin. In the present study, *IREB2* expression was elevated in PBMC of asthmatics after stimulation with rDer p 1. It may be possible that these changes also occur after Der p 1-induced effects on the airways and hence may be involved in bronchial hyperreactivity.

rDer p 1 was found to induce *PPARG* and *HDAC2* expression in asthmatics when compared with changes in-

duced in healthy controls. Both genes are known to be involved in the process of attenuating inflammation rather than its promotion. Generally, *HDAC2* expression is thought to be decreased in asthmatics. We observed this trend in *HDAC2* protein analysis. The activation of the expression of both genes may be part of a protective mechanism which aims to balance the inflammatory response which is also potentially dangerous to the cell.

The fifth group of genes related to airway remodeling analyzed consisted of *ADAM-33*, *CHI3L1* and *MMP9*. All the tested agents influenced the expression of the *CHI3L1* gene, but a significantly higher level of mRNA was observed in asthmatics after Der p 1 stimulation in comparison to healthy controls. Also, relative protein expression was up-regulated. Our findings are in agreement with those of Kuepper et al. [66], who measured elevated YKL-40 concentrations in the serum and bronchoalveolar lavage fluid of asthmatics after segmental allergen challenge. Similarly, Lee et al. [67] observed elevated levels of YKL-40 after an allergen bronchial provocation test with Der f 1 in induced sputum but not in the serum of asthmatic patients. Although the mechanism underlying the interaction between allergens and *CHI3L1* remains unclear, one pathway may involve AP-1 and NF- κ B transcription factors, as it has been established that Der p 1 acts by AP-1 and NF- κ B [50, 68], and YKL-40 expression is AP-1 and NF- κ B dependent [69, 70].

The results of the present study indicate that rDer p 1 is a more potent inducer of gene expression than rFel d 1. After stimulation with rFel d 1, no significant differences in expression were observed between asthmatics and healthy controls (additional data are presented as online suppl. materials, www.karger.com/doi/10.1159/000370067). However, two factors could exert an influence on these findings: the low number of study participants and the reduced ability of rFel d 1 to change the expression of the studied genes in PBMC.

Our study has some limitations. We employed PBMC to study allergen-/LPS-specific responses. They are easily accessible and widely used in in vitro studies, which is in contrast to airway tissue or lavage samples [71]. However, it has been noted that technical aspects of blood sampling, isolation of PBMC, RNA isolation techniques and time to analysis and temperature during blood processing may affect gene expression patterns [72, 73]. The PBMC population is heterogeneous. It includes lymphocytes, T and B cells (70–90%), NK cells, monocytes (10–30%) and dendritic cells (1–2%). In humans, the frequencies of these populations vary across individuals. It seems rationale to assess the composition of the studied cell samples before

experiments. Lineage-specific markers for T (CD3), B (CD19, CD20) and NK cells (CD56) and monocytes (CD14) may be used [72]. Because multiple markers can be used to identify these compartments, frequencies may vary according to the different markers used.

Conclusions

The study shows that the expression of five genes (*ANXA1*, *LTA4H*, *IL1RL1*, *STAT4* and *SOX6*) in the PBMC of asthmatics, COPD patients and healthy subjects are differentially regulated by a bacterial factor responsible for the exacerbation of both diseases. rDer p 1 was the most potent inducer of the studied genes. Relative

protein expression confirmed the observed differences at mRNA level. The present data may provide a basis for more sophisticated studies addressing differences in the course and exacerbation of asthma and COPD.

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Disclosure Statement

The authors declare that they have no conflicts of interests.

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