



Original research article

Effect of *Dermatophagoides pteronyssinus* extract on the expression of genes involved in inflammation and tissue remodeling by peripheral blood mononuclear cells of allergic asthma patients



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ABSTRACT

Purpose: House dust mite allergy constitutes a risk factor for asthma development and is associated with a faster decline of lung function in allergic asthmatics (AAs).

To evaluate the effect of *Dermatophagoides pteronyssinus* (*Dp*) allergens on the expression of genes involved in inflammation and tissue remodeling by peripheral blood mononuclear cells (PBMCs) isolated from the blood of AAs.

Materials and methods: The cells from AAs, allergic rhinitis without asthma patients (ARs), and healthy controls (HCs) were cultured in the presence of *Dp*, lipopolysaccharide (LPS), or without any stimulation. The expression of 84 genes was evaluated using a low-density microarray whereas, the quantitative expression analysis of selected genes was performed using a real-time polymerase chain reaction. The concentration of selected proteins in the cell culture supernatants was assessed using ELISA.

Results: Stimulation of PBMCs with *Dp* and LPS resulted in a significant upregulation of 8 and 15 among 84 studied genes, respectively. The greatest upregulation was observed for *CCL2* and *CCL3* using *Dp* and LPS, respectively. In comparison with HCs, in AAs, significantly increased upregulation of *CCL2* in response to *Dp* was found. The secretion of *CCL2* and *CCL3* by PBMCs reflected the pattern of gene expression at the mRNA level. The mean *Dp*-stimulated secretion of *CCL2* by PBMCs of ARs was less than in AAs ($p < 0.01$), both being notably greater than in the HCs ($p < 0.01$).

Conclusion: Rapid and potent upregulation of *CCL2* expression by PBMCs in response to *Dp* may constitute an important contribution to the development of allergic asthma.

1. Introduction

Allergy to house dust mites (HDMs) constitutes one of the most important risk factors for the development of asthma [1]. Greater exposure of HDM-allergic asthma patients to the house dust is associated with more severe asthma course and is reflected by greater airway hyper-responsiveness (AHR), more frequent asthma exacerbations as well as greater impairment of the lung function [2,3].

However, it is difficult to demonstrate that the avoidance measures, which reduce the environmental exposure to HDMs, are solely responsible for beneficial changes in the health outcomes among patients with HDM-allergic asthma [4]. Usually, the avoidance measures lead to slow

and gradual, however incomplete improvement of the lung function in HDM-allergic asthma patients [5,6]. Moreover, it is more difficult to demonstrate the potential beneficial effects of allergen avoidance in those individuals with a longer duration of the disease [5,6]. This indicates that in the airways of allergic asthma patients (AAs) upon repeated exposures to HDM allergens, some irreversible changes occur. Indeed, deposition of extracellular matrix (ECM) components and sub-epithelial fibrosis are hallmarks of asthma, including HDM-allergic asthma [7]. Those can be found in mild, moderate, and severe asthmatics and the thickness of the subepithelial layer correlates with the asthma severity score, lung function, and AHR [8]. In children with asthma, an association between the level of serum IgE specific to one of

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the HDM species *Dermatophagoides pteronyssinus* (Dp) and basement membrane thickness was demonstrated [9]. Experimental studies provided further evidence indicating the important role of HDM allergy in the process of airway remodeling [10–13]. In mice, exposure to HDM allergens leads to inflammation and remodeling of the airways resembling those detected in asthmatic patients [10]. The direct effect of HDM allergens on the airway epithelium which results in epithelial-mesenchymal transition is well documented [11]. The potential effect of inflammatory cells in HDM-induced airway remodeling has recently gained more interest. Evaluation of the gene expression in the lungs of animals and humans exposed to HDM allergens revealed an up-regulated expression of several genes including those specific for blood-derived mononuclear cells, such as *CCL26* or *CD36* [12]. Moreover, in AAs patients allergen challenge leads to the enhanced influx of circulating monocytes which are crucial for fuelling airway inflammation and amplification of airway remodeling [13]. Similarly, in the experimental model of allergic asthma, bone marrow-derived peripheral blood monocytes participate in the development of airway inflammation and remodeling [13,14]. However, monocytes that are attracted to the airways under certain conditions may be protective and may inhibit the development of HDM-induced asthma in mice [15]. In asthmatic patients, markers of anti-inflammatory monocyte/macrophage activation are detected in induced sputum and can be further modulated by inhaled corticosteroid therapy [16].

Therefore, our study aimed to evaluate the potential in vitro effect of HDM extract on the expression of genes involved in inflammation and tissue remodeling by peripheral blood mononuclear cells in the airways of asthmatic patients.

2. Materials and methods

2.1. Study Design

The study involved 42 allergic rhinitis with asthma patients (AAs), 28 allergic rhinitis without asthma patients (ARs), and 14 nonatopic healthy controls (HCs). Patients were diagnosed by experienced allergists as previously described [17]. In all AAs typical medical history was supported by pulmonary function tests including demonstration of reversibility of airway obstruction or significant bronchoconstrictive response to histamine. Those included in the study were treated with inhaled anti-asthma medications including short-acting-beta agonists (SABA) and inhaled corticosteroids (ICS) with or without long-acting beta-agonist (LABA). All patients who had been treated with allergen immunotherapy before the initial visit were not included in the study. Other exclusion criteria included respiratory tract infection within 3 months before the study, smoking, or any systemic disease.

Allergy status was evaluated using the skin prick testing with a screening panel of aeroallergens (Allergopharma, Reinbek, Germany) as was described earlier [17]. Additionally, the serum concentration of allergen specific IgE (sIgE) was evaluated using the ImmunoCap (Phadia, Uppsala, Sweden). Sensitization to individual major Dp components Der p 1 and Der p 2 was evaluated using a multiparametric allergen test Polychex (Biocheck GmbH, Munster, Germany) according to the manufacturer's instructions.

Lung function testing and exhaled nitric oxide (FeNO) concentration were evaluated as was previously described [18].

2.2. Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized venous blood samples drawn between 7 and 9 a.m. after fasting for at least 8 h. Twenty millilitres of blood were diluted 1:1 with Hanks' solution overlaid on Histopaque 1077 (Sigma-Aldrich, St Louis, MI, USA) and centrifuged at 400×g for 30 min at room temperature. The cells from the Histopaque-plasma interface were collected and after third washing in phosphate-buffered saline (PBS) were resuspended in

complete RPMI1640 medium supplemented with L-glutamine, 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (all Sigma-Aldrich, St Louis, MI, USA). Total cell count and cell viability were evaluated using the Fuchs-Rosenthal counting chamber after mixing the cell suspension with trypan blue at a ratio of 9:1. All cell cultures were run in duplicate. The cells were cultured in the presence of purified Dp extract (Allergopharma, Reinbek, Germany) containing group 1 and group 2 major allergens at a final concentration of 10 and 35 mcg/ml, respectively. Lipopolysaccharide (LPS; Sigma-Aldrich, St Louis, MI, USA) was used at a final concentration of 10 mcg/ml. The exposure time of the cells to either Dp extract or LPS was 6 h and 24 h for RNA and protein assays, respectively. For studies evaluating the effect of different Dp or LPS concentrations, appropriate dilutions were used. In the experiments with individual Dp allergens, natural Der p 1 and Der p 2 (both from Indoor Biotechnologies, Cardiff, UK) were used at a final concentration of 30 mcg/ml. The impact of endotoxin possibly contaminating Dp extract was addressed in the studies with endotoxin inhibitor Polymyxin B (Supplementary Fig. S1).

2.3. Biochemical and immunological assays

The content of the major group 1 and group 2 allergens in the Dp extract was evaluated using ELISA kits (EL-DP1 and EL-D2, respectively; both from Indoor Biotechnologies, Cardiff, UK) according to the manufacturer's instruction, as was described previously [19].

RNA isolation was performed using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instruction [19]. The RNA isolation was performed only in AAs and HCs.

The evaluation of the RNA concentration was performed using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the evaluation of RNA integrity was performed using RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA, USA) and the results were read using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples of high quality (RNA integrity number - RIN>8) were used for further analysis.

Reverse transcription with the random primers was performed on 600 ng of RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Subsequently, the products of reverse transcription were amplified using an SYBR Green-based, 384-well, low-density microarray Human Fibrosis RT² Profiler™ PCR Array (Qiagen GmbH, Hilden, Germany) containing probes allowing for detection of expression of 84 genes involved in the regulation of inflammation and tissue remodeling (Supplementary Table S1).

Equal amounts of the reverse transcription reaction products from 10 samples derived from cultures run in the same conditions were added to the RT2 SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD, USA). Then, the mixture was loaded on the microarray plate in duplicates covering 192 wells containing probes for 84 studied genes and 12 controls including 5 housekeeping genes, genomic DNA contamination control, and PCR control. The probes for each gene were located in four individual wells allowing comparison on a single 384-well plate of samples previously obtained from cultures carried out under two different experimental conditions. Both cultures were run in duplicate. Each plate contained samples from the cell cultures unstimulated and stimulated with either Dp or LPS. Samples derived from AAs and HCs were run on separate plates. The reaction was performed using ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA, USA) and the following thermal cycle profile: initial denaturation for 10 min at 95 °C, then 40 cycles each 15 s at 95 °C and 60 s at 60 °C.

Taqman-based RT-PCR was used for the quantitative evaluation of the expression of the selected genes: CCL2 Chemokine (C–C motif) ligand 2 Hs.303649, NM_002982.3, CCL3 Chemokine (C–C motif) ligand 3 Hs.514107 NM_002983.2, and a housekeeping gene B2M Beta-2-microglobulin Hs.534255, NM_004048.2. The reaction was performed in a 384-well plate in a final volume of 10 µl per reaction using ABI Prism 7900 H T (Applied Biosystems, Carlsbad, CA, USA) with the following

thermal cycle profile: initial denaturation 10 min at 95 °C, then 40 cycles each 15 s at 95 °C and 60 s at 60 °C. *CCL2* and *CCL3* genes were selected based on the greatest upregulation of their expression detected during analysis of low-density microarray results in both examined groups - namely HCs and AAs.

Quantitative assessment of the gene expression was performed using the comparative cycle threshold (Ct) method. The Ct shows the number of cycles at which the amount of amplified target reaches an exponential phase of amplification. For comparative purposes, a Ct of an individual gene is normalized to a Ct of a housekeeping gene and the result is called Δ Ct. Next, the difference between unstimulated and stimulated samples ($\Delta\Delta$ Ct) is calculated by subtracting the Δ Ct of the former from the Δ Ct of the latter. The fold change was determined using the representative formula: $2^{-\Delta\Delta$ Ct.

The concentration of *CCL2* and *CCL3* in the cell culture supernatants was evaluated using Quantikine ELISA Kits (R&D Diagnostics, Minneapolis, MO, USA) according to the manufacturer's instruction. All assays were run in duplicate.

The concentration of total and *Dp*-specific IgE in serum was evaluated using ImmunoCAP (Phadia, Uppsala, Sweden).

2.4. Statistical Analysis

The distribution of individual parameters was evaluated using the Kolmogorov-Smirnov test. Normally distributed continuous variables were presented as means with the standard deviations (SD) and compared using the ANOVA test. The post-hoc analysis was performed using the Student's t-test. Not-normally distributed continuous variables were presented as medians with 95% confidence intervals (95% CI) and compared using the Kruskal-Wallis test. The post-hoc analysis was performed using the Wilcoxon test. Values of $p < 0.05$ were considered to be statistically significant. All computations were carried out using the Statistica software (StatSoft, Krakow, Poland).

2.5. Ethical issues

The study was approved by the local Bioethics Committee of the Medical University of Białystok (approval number: R-I-002/286/2008). All participants provided written informed consent.

This study which involves human participants is in compliance with the 1964 Helsinki declaration and its later amendments.

3. Results

The characteristics of the studied patients are presented in Table 1. The groups did not differ statistically significantly in age and sex distribution. Asthmatic patients were characterized by lower lung function parameters and elevated FeNO levels. In all allergic patients significant sensitization (IgE > 0.7 kU/L) to major *Dp* allergens - Der p 1 and Der p 2, was demonstrated and the median IgE serum concentrations were not

different between AAs and ARs (Table 1).

The experiments with an endotoxin inhibitor Polymyxin B confirmed that the extract used in the study did not contain sufficient level of endotoxin to affect the results (Supplementary Fig. S1).

Analysis of the SYBR Green-based low-density microarray results demonstrated that among the 84 evaluated genes a significant (>2-fold) upregulation of *CCL2*, *CCL3*, *IL-1b*, *MMP-14*, and *TSP-1* expression was observed in *Dp*-stimulated PBMCs of AAs and HCs (Supplementary Table S2). Considerable upregulation of *IL-10*, *IL-13*, and *MMP-9* was detected exclusively in AAs but not in HCs (Supplementary Table S2). The most prominent upregulation was demonstrated for *CCL2* in both AAs and HCs. Stimulation of PBMCs with LPS induced notable upregulation of *CCL2*, *CCL3*, *IFN-γ*, *IL-1a*, *IL-1b*, *IL-10*, *Integrin b3*, *Integrin b8*, *MMP-3*, *MMP-14*, *NFKB1*, *STAT-1*, *TNF-α*, *TSP-1*, and *V-myc* expression in AAs. In LPS-stimulated PBMCs from HCs significant upregulation of the same genes, except *Integrin b3*, *MMP-3* and *V-myc* were demonstrated (Supplementary Table S2) whereas the most noticeable upregulation was demonstrated for *CCL3* in both AAs and HCs.

Quantitative evaluation of *CCL2* and *CCL3* expression using Taqman-based RT-PCR confirmed the results obtained with the microarray. In both, HCs and AAs, 6-h stimulation with *Dp* resulted in statistically significant upregulation of *CCL2* (11.8-fold, 95%CI 6.9–15.2-fold, $p < 0.001$, and 18.6-fold, 95%CI 14.7–21.9-fold, $p < 0.001$, respectively) and *CCL3* (4.3-fold, 95%CI 3.3–6.2-fold, $p < 0.001$, and 3.5-fold, 95%CI 2.5–4.8-fold, $p < 0.001$, respectively) expression (Fig. 1). The upregulation of *CCL2* expression by *Dp* was statistically significantly greater than that of *CCL3* in both HCs ($p < 0.001$) and AAs ($p < 0.001$). Stimulation of PBMCs with *Dp* produced in AAs statistically significantly greater upregulation of *CCL2* expression than in HCs ($p < 0.001$) (Fig. 1).

On the contrary, 6-h stimulation of PBMCs with LPS resulted in a more robust upregulation of *CCL3* than *CCL2* expression both in HCs (22.5-fold; 95%CI 15–34.4-fold vs. 7.8-fold; 95%CI 4.4–12.7-fold; $p < 0.001$) and AAs (21-fold; 95%CI 18–25.4-fold vs 8.2-fold; 95%CI 6.9–9.6-fold; $p < 0.0001$) (Fig. 1).

The secretion of *CCL2* and *CCL3* by PBMCs was assessed in the cell cultures derived from HCs, ARs, and AAs. Furthermore, in unstimulated PBMC cultures the mean concentration of *CCL2* was statistically significantly greater in AAs (82.9 ± 46.4 pg/ml) than in ARs (49.1 ± 17.7 pg/ml; $p = 0.001$) both being statistically significantly higher than in HCs (32.4 ± 21.4 pg/ml; $p = 0.001$ and $p = 0.01$, respectively). The mean *CCL3* concentration in supernatants of unstimulated PBMCs did not differ between AAs (38.0 ± 32.5 pg/ml), ARs (35.8 ± 19.9 pg/ml), and HCs (29.1 ± 17.8 ; $p = 0.563$) (Fig. 2). Moreover, in AAs, the mean concentration of *CCL2* was statistically significantly greater than that of *CCL3* ($p < 0.001$) (Fig. 2).

Stimulation of PBMCs with *Dp* or LPS resulted in an increase of *CCL2* and *CCL3* secretion which was consistent with the changes in *CCL2* and *CCL3* expression noticed at the mRNA level. In *Dp*-stimulated cultures the mean concentration of *CCL2* was statistically significantly higher than that of *CCL3* in HCs (3451 ± 1891 pg/ml; vs 906 ± 770 pg/ml; $p <$

Table 1
The clinical characteristics of the examined groups of patients.

	AAs (n = 42)	ARs (n = 28)	HCs (n = 14)
Age (years)	27; 24.3 to 31.4	25; 19 to 33	26; 21.3 to 32
Sex (M/F)	24/18	16/12	8/6
FEV1 (% predicted)	89; 80.7 to 98.4 #, ^	99; 84 to 112	103.5; 93.7 to 114
MEF50 (% predicted)	65.3; 53–78.7 #, ^	92; 76–98	96.5; 88.3 to 101.7
FeNO (ppb)	50; 38 to 63 &, *	33; 26 to 48	11; 7 to 15
Total IgE (kU/L)	206; 154 to 318	178; 96 to 301	ND
Anti- <i>Dp</i> IgE (kU/L)	10.9; 6.5 to 20.7	7.8; 4.4 to 18.9	ND
Anti-Der p 1 IgE (kU/L)	4.53; 2.29 to 8.29	3.9; 1.6 to 5.89	ND
Anti-Der p 2 IgE (kU/L)	10.19; 5.33 to 13.4	8.85; 6.95 to 12	ND
ICS (number of patients)	24 (57.1%)	NA	NA

Abbreviations: AA – allergic asthmatics, AR – patients with allergic rhinitis without asthma, HCs – healthy controls, # - significantly less than in HCs, ^ - significantly less than in ARs, & - significantly greater than in HCs, * - significantly greater than in ARs, ND – not done, NA - ? not applicable, ICS - ? inhaled corticosteroids.

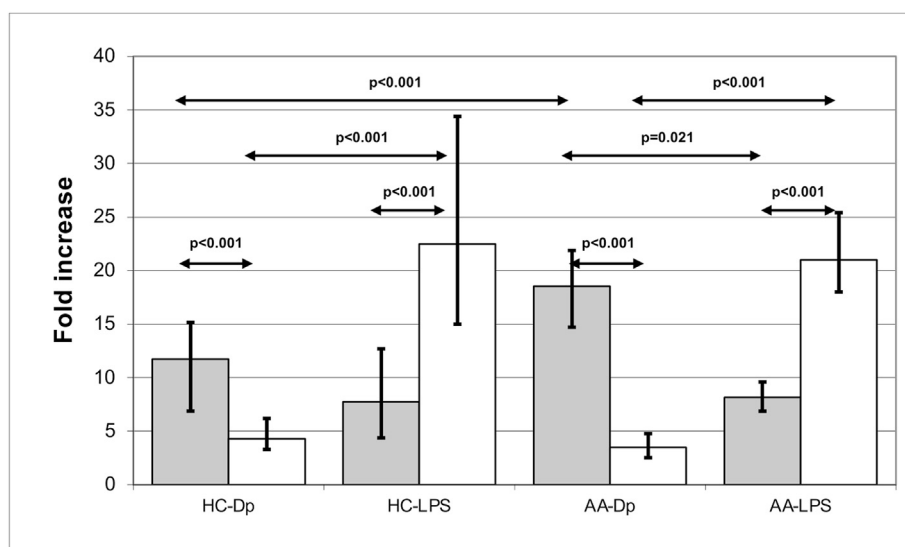


Fig. 1. The expression of *CCL2* and *CCL3* on mRNA level by peripheral blood mononuclear cells (PBMCs) of healthy controls (HC) and allergic asthma (AA) patients stimulated with *Dermatophagoides pteronyssinus* extract (Dp) or lipopolysaccharide (LPS). The bars represent median values with 95% CI; Grey bars – *CCL2*; White bars – *CCL3*. Abbreviations: *CCL2* – gene of chemokine (C–C motif) ligand 2, *CCL3* – gene of chemokine (C–C motif) ligand 3.

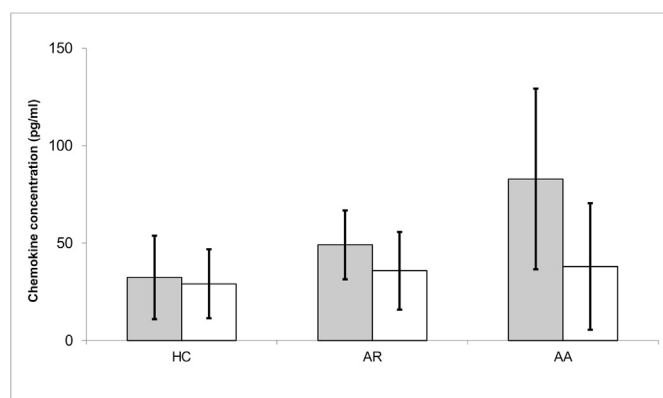


Fig. 2. The concentration of *CCL2* and *CCL3* in supernatants of unstimulated peripheral blood mononuclear cell (PBMC) cultures of healthy controls (HC), patients with allergic rhinitis without asthma (AR), and allergic asthmatics (AA). Median ($\pm$ 95% CI) concentration of *CCL2* – grey bars or *CCL3* – white bars in supernatants of PBMC cultures. Abbreviations: *CCL2* – chemokine (C–C motif) ligand 2, *CCL3* – chemokine (C–C motif) ligand 3.

0.001), ARs (5588 ± 1879 pg/ml vs 1032 ± 604 pg/ml; $p < 0.001$) and AAs (8742 ± 3639 pg/ml; $p < 0.001$) (Fig. 3). On the contrary, in the cell cultures stimulated with LPS the mean concentration of *CCL3* was statistically significantly greater than that of *CCL2* in HCs (31528 ± 11243 pg/ml vs 4530 ± 2853 pg/ml; $p < 0.001$), ARs (31159 ± 6873 pg/ml vs 5241 ± 2965 pg/ml; $p < 0.001$) and AAs (32482 ± 10282 pg/ml vs 5605 ± 2440 pg/ml; $p < 0.001$). The mean *CCL2*/*CCL3* ratio in Dp-stimulated PBMC culture supernatants of HCs ($529 \pm 349\%$), AR ($800 \pm 646\%$) and AAs ($1075 \pm 867\%$) were statistically significantly greater than those in the corresponding LPS stimulated PBMC supernatants ($14.5 \pm 8.5\%$; $p < 0.001$, $17.7 \pm 12.2\%$; $p < 0.001$, and $18.1 \pm 8.5\%$; $p < 0.001$; respectively for HCs, ARs and AAs) (Fig. 4).

The secretion of *CCL2* and *CCL3* by Dp or LPS stimulated PBMCs was dose-dependent (Fig. 5). Stimulation with Dp resulted in an intensified secretion of *CCL2* rather than *CCL3* by PBMCs across a broad range of Dp concentrations. At low Dp concentrations, a statistically significant increase was found in *CCL2* concentration with no statistically significant increase in *CCL3* concentration (Fig. 5). This remains in contrast to LPS stimulation which led to a greater increase in *CCL3* than in *CCL2* secretion. Finally, in vitro stimulation of PBMCs with purified Der p 1, Der p 2 or Der p 1 + Der p 2 resulted in a pattern of *CCL2* and *CCL3* production similar to that of the whole extract (Supplementary Fig. S2). However,

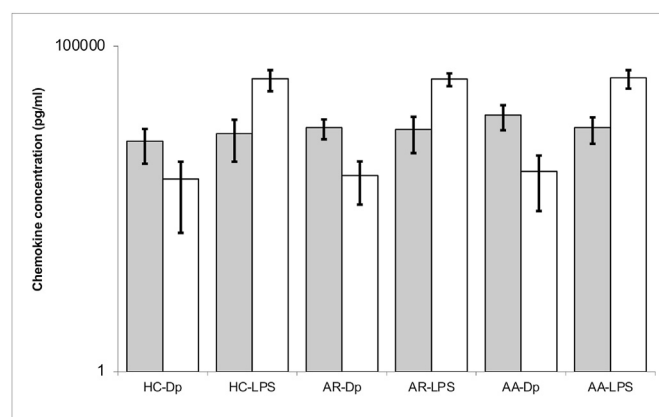


Fig. 3. The concentrations of *CCL2* and *CCL3* in supernatants of peripheral blood mononuclear cell (PBMC) cultures of healthy controls (HC), patients with allergic rhinitis without asthma (AR), and allergic asthmatics (AA) stimulated with Dp or LPS. Median ($\pm$ 95% CI) concentration of *CCL2* – grey bars or *CCL3* – white bars in supernatants of PBMC cultures. Abbreviations: *CCL2* – chemokine (C–C motif) ligand 2, *CCL3* – chemokine (C–C motif) ligand 3, Dp – *Dermatophagoides pteronyssinus*, LPS – lipopolysaccharide.

the concentration ratio of *CCL2* to *CCL3* was less than that revealed in the cell cultures stimulated with the whole allergen extract.

4. Discussion

Environmental exposure to Dp is a risk factor for the development of allergic asthma while exposure to LPS may be protective factor [2,3,20]. In this study, we evaluated the profile of gene expression induced by Dp in PBMCs of HCs and AAs and compared it to that induced by LPS. We focused on the genes involved in the regulation of inflammation and tissue remodeling as those processes are crucial for the development of asthma phenotype in allergic patients [7,9–11]. Using this approach, we searched for a candidate gene(s) whose expression is regulated differently by Dp and LPS stimulation. Our results indicated that Dp and LPS induced different patterns of gene expression in PBMCs in vitro. Moreover, we were able to show that the gene expression profile was altered in AAs in comparison with HCs. The most apparent induction was demonstrated for *CCL2* and *CCL3* expression in PBMCs stimulated with Dp and LPS, respectively. Both *CCL2* and *CCL3* belong to the same family of

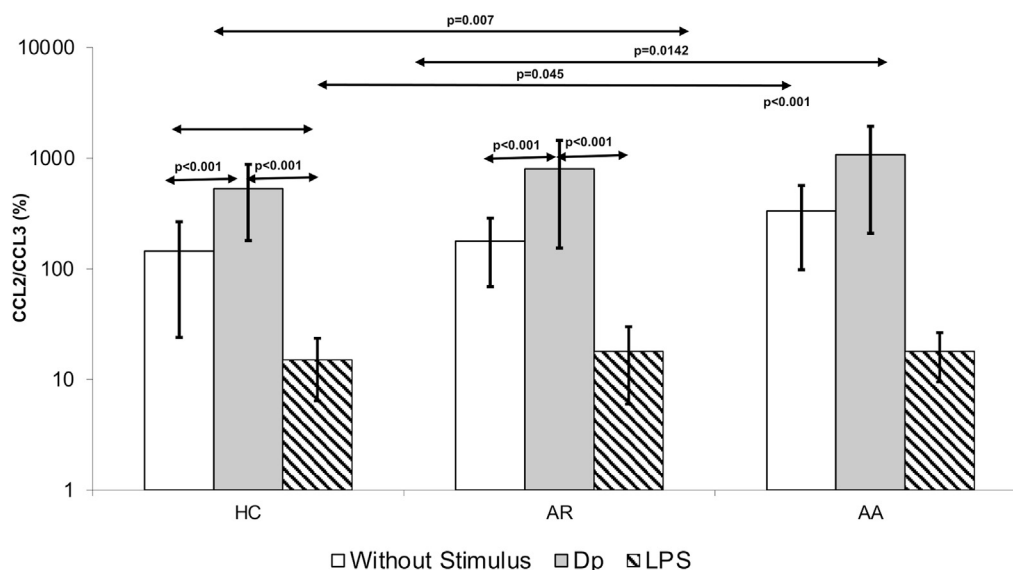


Fig. 4. The ratio between CCL2 and CCL3 concentration in the supernatants of PBMCs cultured for 24 h in the presence of *Dp*, LPS or without any stimulation.

Median ($\pm 95\%$ CI) ratio of CCL2 – grey bars and CCL3 – white bars in supernatants of PBMC cultures. Abbreviations: CCL2 – chemokine (C–C motif) ligand 2, CCL3 – chemokine (C–C motif) ligand 3, HC – healthy controls, AR – patients with allergic rhinitis without asthma, AA – allergic asthmatics, *Dp* – *Dermatophagoides pteronyssinus*, LPS – lipopolysaccharide.

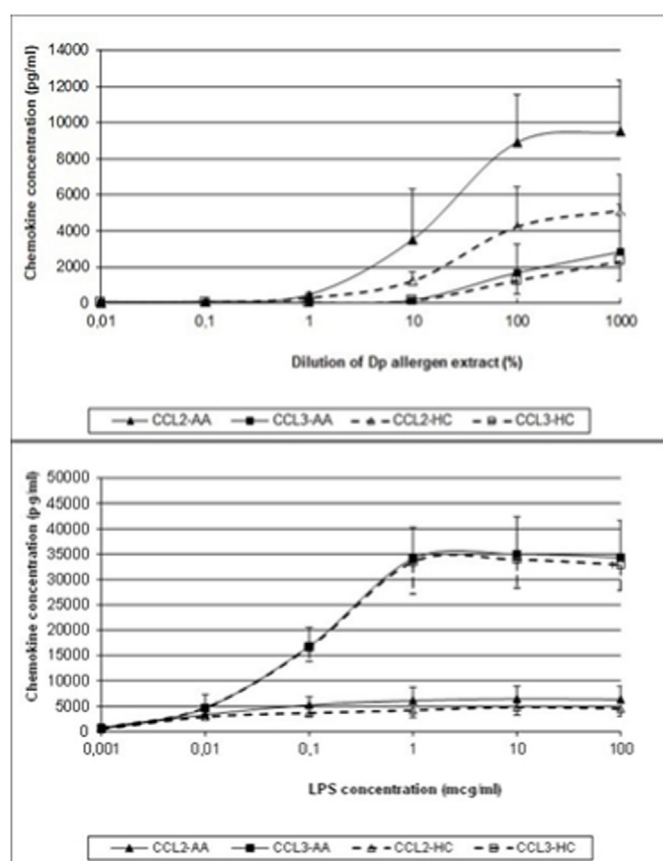


Fig. 5. The effect of different concentrations of *Dp* or LPS on the production of CCL2 and CCL3 by peripheral blood mononuclear cells (PBMCs) of healthy controls (HC) and allergic asthmatics (AA).

Abbreviations: CCL2 – chemokine (C–C motif) ligand 2, CCL3 – chemokine (C–C motif) ligand 3, *Dp* – *Dermatophagoides pteronyssinus*, LPS – lipopolysaccharide.

CC-chemokines, but they act on different receptors and therefore, exert different effects on immune response, inflammation, and tissue remodeling [21]. Thus, we decided to evaluate the effect of *Dp* and LPS on the expression of these chemokines in more details. The quantitative assessment of CCL2 and CCL3 expression in PBMCs using Taqman-based

RT-PCR confirmed the results of the microarray method pointing out that among the studied 84 genes CCL2 was the most potently up-regulated by *Dp* stimulation. Moreover, an increase of CCL2 and CCL3 concentration in the *Dp*- and LPS-stimulated cell culture supernatants corresponded to the changes of the aforementioned genes expression detected on the mRNA level. The results of our study, which indicated that the dominant CCL2 upregulation by *Dp*-stimulated PBMCs, are consistent with those noted after the in vitro stimulation of a monocytoic cell line THP-1 by *Dp* [22]. Whole *Dp* extracts rapidly induced CCL2 synthesis in THP-1 cells and the effect was independent of its proteolytic activity [22]. Similarly, stimulation of PBMCs derived from allergic asthma patients with a major *Dp* allergen Der p 1 caused the upregulation of CCL2 [23]. Interestingly, in Der p 1-induced PBMCs derived from HDM AAs the fractional increase of CCL2 secretion was more than 10-times greater than that of CCL5 or CXCL8 [23]. This study remains consistent with our observations which reveal that CCL2 is the most potently up-regulated in *Dp*-stimulated PBMCs. However, we were also able to demonstrate that in comparison with HCs, *Dp*-induced CCL2 expression was augmented in PBMCs of AAs. Stimulation of THP-1 cells or PBMC-derived macrophages with LPS results in the induction of many nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) dependent genes and CCL3 is one of the most potently up-regulated [24]. Those findings are consistent with our results, which indicate that the expression of CCL3 in PBMCs is highly responsive to LPS stimulation.

The effects of in vivo allergen challenge on the CCL2 secretion in humans and mice are similar to those obtained in vitro [25–27]. In AAs, increased local production of CCL2 was detected already 4 h after the endobronchial allergen challenge [25]. Moreover, the whole lung allergen challenge of HDM AAs patients, which led to the development of an asthmatic response, resulted in a significant increase of CCL2 plasma concentration within 24 h [26]. Interestingly, in a mouse model of AAs CCL2 derived from bronchial macrophages and epithelial cells played a major role in the development of airway inflammation and AHR [27]. Similarly, in humans, macrophages play a pivotal role in allergen induced CCL2 production. Evaluation of the chemokine production by the cells obtained using bronchoalveolar lavage (BAL) showed that these obtained from allergen challenged airways produced ex vivo more CCL2 than those obtained from saline challenged ones [25]. Interestingly, no effect of allergen challenge on CCL3 production by BAL fluid cells could be demonstrated [25]. This supports our observations showing the predominant upregulation of CCL2 in *Dp*-stimulated PBMCs. The differences in the chemokine expression seem to reflect specific regulation of CCL2 and CCL3 expression by *Dp* and LPS. They are not likely to depend solely

on the potency of individual stimuli as predominant secretion of CCL2 and CCL3 persisted along with a broad range of *Dp* and LPS concentrations used for stimulation of PBMCs, respectively.

Interestingly, both *CCL2* and *CCL3* genes are located nearby on the long arm of chromosome 17 (chr.17, q11.2) [21]. Although they share some similar effects on leukocyte trafficking and tissue remodeling, they differ in their effects on the T cell differentiation and activation [21]. In a mouse model, it has been demonstrated that CCL2 plays a crucial and indispensable role in the generation of Th2-type immune response [28].

Moreover, CCL2 but not CCL3 directly activates basophils for histamine release [29]. Furthermore, blockade of CCL2/CCR2 signaling pathway attenuated ovalbumin-induced allergic asthma in mice [30]. Therefore, the dominant upregulation of *CCL2* expression induced by *Dp* in PBMCs of AAs indicates possible involvement of this pathway in the pathogenesis of allergic asthma.

However, in our study, elevated secretion of CCL2 by PBMCs of allergic patients could be demonstrated also spontaneously. Consistently, an elevated level of CCL2 was shown in BAL fluid and bronchial tissue of asthmatic patients [31]. This raises the hypothesis that mechanisms controlling *CCL2* expression may be dysregulated in AAs. Indeed, genetic, and epigenetic mechanisms that affect the production of CCL2 have already been described in allergic patients [32,33]. The presence of single nucleotide polymorphisms in the regulatory region of *CCL2* which affect the chemokine expression was demonstrated in AAs [31]. Histone deacetylase 3 (HDAC3), an enzyme involved in epigenetic regulation of gene expression, was shown to affect the skin allergic reaction by regulating *CCL2* expression [33]. Moreover, Th-2 cytokines may promote an increased *CCL2* expression by mononuclear phagocytes. Pre-incubation of THP-1 cells with IL-4 promoted *CCL2* upregulation in response to cysteine leukotriene 1 receptor activation [34]. A similar mechanism may operate also in allergic patients leading to elevated spontaneous and *Dp*-induced CCL2 secretion.

Our study has been restricted to the evaluation of the whole allergen extract and two major allergens - Der p 1 and Der p 2. However, other HDM allergens may play a role in modulating inflammatory response and developing asthma phenotype in HDM allergic patients [16,35].

Demonstrated in this study predominant secretion of CCL2 by *Dp*-stimulated PBMCs revealed that chemokine may be an interesting therapeutic target in the therapy of allergic diseases, including allergic asthma. Further studies are warranted to dissect the role of individual receptors and signal transduction pathways in induction/regulation of individual gene expression by HDM allergens.

4.1. Limitations and strengths of the study

The main limitation of our study is evaluation of a restricted number of transcripts. Application of RNA sequencing (RNA-Seq) methods could have revealed additional genes whose expression is differentially regulated in response to *Dp* or LPS. This method has recently been used in the evaluation of transcriptional profile which might be more comprehensive than microarrays especially for the identification of new or alternative transcripts [36]. However, the main aim of this study was to analyse the expression of widely known genes involved in inflammation and lung tissue remodeling, processes which are crucial for the development of asthmatic phenotype in allergic patients. Nonetheless, using this limited approach we were able to successfully single out genes whose expression was differentially regulated by allergen extract and endotoxin. Moreover, we managed to demonstrate that in asthmatic patients the transcriptional mechanisms of *CCL2* expression are dysregulated which in turn resulted in enhanced release of the chemokine upon allergen challenge.

Other concern might be related to the cell lines used in the study. PBMCs although consisting of various cells, constitute an easily accessible source of human immune cells and accurately reflect the immunological relations occurring in the human body. Those cells have been used in many other clinical studies in the field of allergy research.

Furthermore, an important concern might be associated with the

presence of gut microbiota in the *Dp* extracts [37,38]. Pascoe et al. [37] in their animal experiments underlined the importance of defining the LPS content in HDM allergen extracts to provide high reproducibility and reliability of the study. Moreover, they indicated that endotoxin content differs dramatically in HDM extracts derived from different sources, indicating that different methods of protein extraction may result in allergen extract more or less contaminated by endotoxin [37]. Interestingly, a recent study conducted by Kim et al. [38] showed that concerns about the involvement of microbiome in immunological responses on the HDM allergen extracts might be referred to *Dermatophagoides farinae* extracts but less so in case of *Dp* extracts. It was shown that *Dp* extracts contained antimicrobial substances produced by symbiotic mycobiome, thereby, significantly decreasing the likelihood of *Dp* extract contamination with bacterial-derived-endotoxin [38]. Our experiments indicated that the *Dp* extract used in our study did not contain endotoxin at a level which could significantly influence the results. Therefore, applying such a highly purified *Dp* extract allowed to differentiate the effect of the allergen from that of the endotoxin on expression of the selected genes by PBMCs. However, concomitant exposure to endotoxin and allergens is likely in asthmatic patients. Therefore, future research on the potential influence of bacterial products including gut microbiota on response to allergen exposure in asthma patients is strongly warranted.

5. Conclusions

In summary, our study revealed that *CCL2* and *CCL3* belong to the most potently induced genes by *Dp* and LPS, respectively. Peripheral blood monocytes constitute a major source of those chemokines. Moreover, both spontaneous and allergen-induced expression of *CCL2* is enhanced in AAs. Predominant upregulation of *CCL2* by *Dp* may participate in the development of asthma and its progression in HDM-exposed individuals.

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The author contribution

Study Design: Pawel Bernatowicz, Agnieszka Pampuch, Krzysztof Kowal.

Data Collection: Pawel Bernatowicz, Agnieszka Pampuch, Hubert Zywno, Krzysztof Kowal.

Statistical Analysis: Pawel Bernatowicz, Agnieszka Pampuch, Hubert Zywno, Krzysztof Kowal.

Data Interpretation: Pawel Bernatowicz, Agnieszka Pampuch, Hubert Zywno, Krzysztof Kowal.

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Declaration of competing interest

KK received a lecture fee from: ALK Abello, Astra Zeneca, Berlin Chemie, Emma, Hal Allergy, Orion Pharma, TEVA Pharmaceuticals and royalties from UpToDate.

PB, AP and HZ declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.advms.2022.05.002>.

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