



Original Research Article

Soluble CD163 modulates cytokine production by peripheral blood mononuclear cells stimulated by *Dermatophagoides pteronyssinus* allergens in vitroPawel Bernatowicz^a, Krzysztof Kowal^{a,b,*}^a Department of Experimental Allergology and Immunology, Medical University of Białystok, Białystok, Poland^b Department of Allergology and Internal Medicine, Medical University of Białystok, Białystok, Poland

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ABSTRACT

Purpose: The CD163 is a scavenger receptor expressed exclusively on monocytes/macrophages which has been shown to exert anti-inflammatory effects. The aim of this study was to evaluate the effect of exogenous sCD163 on production of selected cytokines by peripheral blood mononuclear cells (PBMC) of house dust mite allergic patients (AAPs) stimulated in vitro with *Dermatophagoides pteronyssinus* (Dp) allergens.

Patients and methods: The study was performed in 24 AAPs and 12 healthy control subjects (HCs). Peripheral blood mononuclear cells were cultured for up to 144 h (T₁₄₄) in the presence of Dp extract with or without sCD163. Concentration of interleukin (IL) – 10, IL-13 and transforming growth factor beta (TGF-β) was evaluated in the cell culture supernatants using ELISA. Expression of the selected cytokines was evaluated in cell culture lysates using Taqman-based real time polymerase chain reaction (RT-PCR).

Results: Dp-stimulated PBMC from AAPs released more IL-10 and IL-13 than those from HCs. The greatest up-regulation of *IL-10* expression was seen at T₆, while that of *IL-13* was delayed. Soluble CD163 augmented production of IL-10 in response to Dp stimulation. No significant effect of sCD163 on production of IL-13 and IL-10 by PBMC of HCs could be demonstrated.

Conclusions: In AAPs sCD163 modulates the immune response to Dp allergens potentiating anti-inflammatory, homeostatic mechanisms.

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1. Introduction

CD163 is a protein of 130 kD molecular weight belonging to the group B of the scavenger receptor cysteine-rich (SRCR) superfamily [1,2]. It is exclusively expressed by mononuclear phagocytes as a type I transmembrane protein [1]. The protein is shed from the cell membrane and as a soluble form (sCD163) is present in body fluids including plasma and sputum [1,3,4]. Expression of CD163 is strongly up-regulated by anti-inflammatory mediators including corticosteroids (CS) and interleukin-10 (IL-10) [5–12]. Corticosteroids are very potent inducers of CD163 expression and the

protein, at least under certain circumstances, participates in anti-inflammatory action of CS [5–7]. Interleukin-10 is also involved in the regulation of CD163 expression in many physiological and pathological conditions [8–12]. In fact, in experiments utilizing gene-chip technology CD163 displays the strongest response to IL-10 among all 19 of the up-regulated genes [10]. Interleukin 10 is also responsible for up-regulation of monocyte/macrophage CD163 expression induced by CD4+CD25+Foxp3+ T regulatory cells and for rebound up-regulation of monocyte/macrophage CD163 expression after shedding of the receptor in response to Toll like receptor (TLR) stimulation [11,12]. Monocytes/macrophages expressing CD163 release broad range of anti-inflammatory mediators including IL-10 and carbon monoxide (CO) [13]. Membrane bound CD163 is responsible for fueling this anti-inflammatory response under certain clinical conditions [13]. Not only membrane CD163 but also sCD163 exerts anti-inflammatory action. Soluble CD163 inhibits in a dose-dependent

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manner phorbol ester-induced T cell activation and proliferation in vitro [14,15]. This anti-inflammatory function of CD163 seems to be restricted to its soluble form, as membrane-bound CD163 does not exert such an effect [16].

The anti-inflammatory effects of CD163 can be also demonstrated in vivo [17]. In particular, in patients with rheumatoid arthritis histological analysis of the inflamed joints revealed that wherever intense T cell proliferation was observed little or no CD163 was seen on tissue macrophages [17]. Moreover, lymphoid follicle macrophages, which are located in a place where intensive lymphocyte proliferation occurs, express little or no CD163 at all [18,19].

It seems that CD163 may also be involved in the regulation of the inflammatory response to allergen challenge. Our previous studies demonstrated that circulating monocytes of asthmatic patients were characterized by greater expression of CD163 than those of healthy subjects [20]. Moreover, those that express high level of CD163 seem to be preferentially attracted to the airways of allergic asthma patients after allergen exposure [21]. The role of CD163 as an inflammatory molecule has been demonstrated in experimental studies using genetically modified CD163 knockout mice [22]. House dust mite-induced airway inflammation was augmented in CD163 knockout mice in comparison to their wild type counterparts. This provides evidence for important in vivo anti-inflammatory effects of CD163 in response to allergen exposure.

It was therefore of interest to investigate in vitro the effect of exogenous sCD163 on production of selected cytokines by peripheral blood mononuclear cells from house dust mite allergic asthma patients stimulated with allergen extract of a house dust mite *Dermatophagoides pteronyssinus* (Dp).

2. Patients and methods

The study was performed in 24 house dust mite allergic asthma patients (AAPs). In addition 12 nonatopic healthy subjects (HCs) were included as a control group. Asthma was diagnosed according to the GINA guidelines [23]. In all patients either forced expiratory volume within the first second (FEV₁) of less than 80% of the predicted value with at least 12% improvement 15 min after inhalation of 400 mcg salbutamol and/or significant bronchoconstrictive response to inhaled histamine was demonstrated. Patients with any other systemic diseases, suffering from respiratory tract infection within 1 month before the study or smoking were not included in the study. The study was approved by the local Bioethics Committee (R-I-002/286/2008). All participants provided written informed consent.

2.1. Skin prick tests

All persons were skin tested using prick methodology with a screening panel of aeroallergens (Allergopharma, Reinbek, Germany) as described earlier [24].

2.2. Bronchial challenge

Histamine bronchial challenge was performed according to the method previously described elsewhere [20]. Briefly, all patients inhaled doubling concentrations of histamine starting from a concentration of 0.62 mg/ml. Aerosol was generated using a DeVilbiss #646 nebulizer attached to a Rosenthal – French dosimeter. All subjects performed five inspiratory – capacity breaths of given histamine concentration. Forced expiratory maneuvers were performed 90 s after each fifth inhalation. The procedure was continued until either at least 20% fall of FEV₁ or histamine concentration 32 mg/ml was reached. Bronchial

reactivity to histamine is expressed as histamine concentration causing 20% fall of FEV₁ (PC₂₀).

2.3. Cell isolation and culture

Peripheral blood mononuclear cells were isolated from 20 ml of venous blood by density gradient centrifugation using Histopaque 1077 (Sigma–Aldrich, St. Louis, MO, USA). Total number and viability of isolated cells were assessed using Fuchs Rosenthal chamber and trypan blue exclusion method. The cells were cultured in 24-well culture plates at a density 1×10^6 cells/ml in RPMI-1640 medium supplemented with 5% heat inactivated fetal calf serum, L-glutamine, penicillin and streptomycin. The cells were cultured in the presence of Dp (Allergopharma, Reinbek, Germany) at a concentration 5000 SBE/ml with or without sCD163 (R&D Systems, Minneapolis, MN, USA) at a final concentration 0.5 µg/ml. In addition, cell cultures without any stimulation was used as a negative control. The final concentrations were established in a preliminary dose-response studies (not shown).

After 6, 12, 24, and 144 h the supernatants were separated from cells, aliquoted and stored frozen at –70 °C until tested. The cells were treated with lysing solution and stored frozen at –70 °C for further isolation of RNA.

2.4. Biochemical and immunologic assays

RNA isolation was performed using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instructions.

The evaluation of 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) according to the manufacturer instructions and the results were read using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Only RNA of high quality (RNA integrity number – RIN > 8) was used for further analysis.

Taqman-based real time PCR was used to quantitatively evaluate expression of the selected genes *IL-10* Hs.193717, NM_000572.2, *IL-13* Hs.845, NM_002188.2, *TGF-β* Hs.645227, NM_000660.4, and a house keeping gene *B2M* Hs.534255, NM_004048.2. The reaction was performed in 384-well plate in a final volume of 10 µl per reaction using ABI Prism 7900HT (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.

Quantitative assessment of gene expression was performed using the comparative threshold cycle (CT) method. The threshold cycle (CT) indicates the number of cycles at which the amount of amplified target reaches an exponential phase of amplification. For comparative purpose a CT of an individual gene is normalized to a CT of a house keeping gene and the result is called ΔCT . Then the difference between unstimulated and stimulated samples ($\Delta\Delta CT$) is calculated by subtracting ΔCT of the former from the ΔCT of the latter. The fold change was determined using formula $2^{-\Delta\Delta CT}$.

Concentration of IL-10 and IL-13 in cell culture supernatants was evaluated using high sensitivity enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN, USA and Abcam, Cambridge, UK, respectively) according to the manufacturer's instruction. All samples were run in duplicates.

2.5. Statistical analysis

Normal distribution was evaluated using Wilk-Shapiro test. Comparison of normally distributed continuous variables was

performed using ANOVA test. Comparison of not-normally distributed continuous variables was performed using the Mann–Whitney *U* test. Data of normally distributed continuous variables were expressed as means with the standard deviations. Data of not normally distributed variables were expressed as medians with 95% confidence intervals (95%CI). All computations were carried out using the Statistica software.

3. Results

3.1. Patient demographics

Patients characteristics is presented in Table 1. All included patients suffered from mild-moderate allergic asthma and were sensitized to house dust mite allergens. There was no difference in age and sex distribution between AAPs and HCs. Significantly

Table 1

Patients characteristics.

	AAPs (n = 24)	HCS (n = 12)	p
Age (years)	25; 20–34	26; 19–36	0.754
Sex (M/F)	14/10	7/5	0.874
FEV1 (% predicted)	87; 75–96	101; 91–113	0.006
Total IgE (kU/L)	174; 109–365	ND	
Anti-Dp IgE (kU/L)	12; 4.3–68.9	ND	
ICS (number of patients)	12 (50%)	NA	
ICS daily dose (μ g) ^a	400; 200–800	NA	

^a Equivalent of budesonide.

ND – not done.

NA – not applicable.

lower lung function parameters were demonstrated in AAPs than in HCs. Allergic asthma patients were characterized by elevated levels of total and *D. pteronyssinus*-specific IgE serum concentrations.

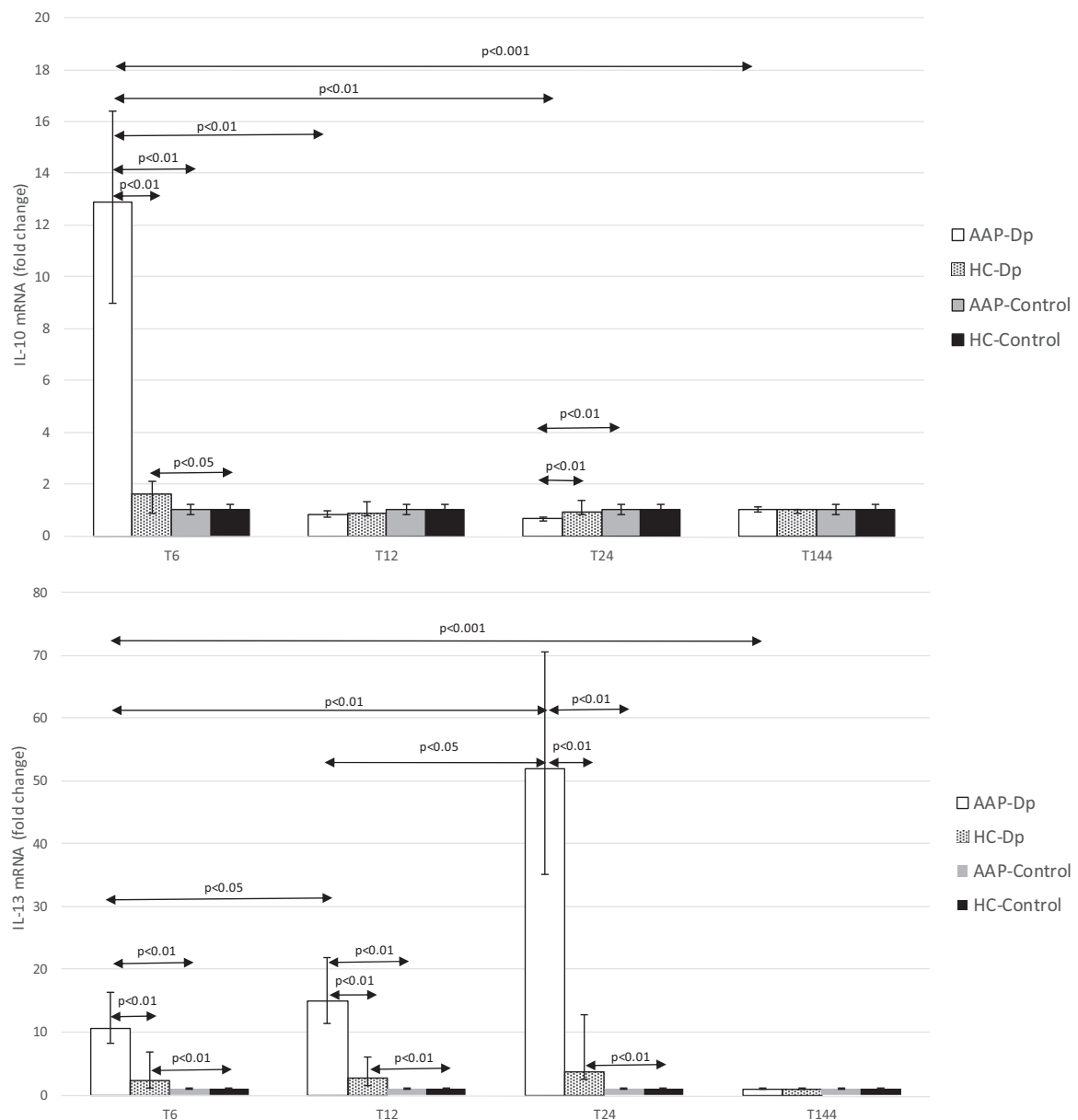


Fig. 1. The effect of Dp on expression of *IL-10* (upper panel) and *IL-13* (lower panel) by PBMC derived from AAP and HC cultured for 6 (T₆), 12 (T₁₂), 24 (T₂₄) and 144 (T₁₄₄) hours. The results are expressed as medians with 95% confidence intervals. The values represent fold change of *IL-10* or *IL-13* mRNA expression by Dp stimulated cells in relation to that by unstimulated.

3.2. Dermatophagoides pteronyssinus allergens enhance cytokine expression predominantly in BMC of AAP

In vitro stimulation of PBMC with Dp up-regulated expression of *IL-10* and *IL-13* at mRNA level (Fig. 1). The kinetics of *IL-10* and *IL-13* expression was different and the response was different in AAPs and HCs. The greatest increase of *IL-10* expression was seen at T_6 (12.9 fold; 95%CI 9–16.4 fold) in PBMCs of AAPs and was significantly greater than that of HCs (1.62 fold; 95%CI 0.85–2.11 fold; $p < 0.01$). Subsequently, *IL-10* expression in Dp-induced PBMCs from AAPs rapidly decreased being at T_{24} significantly lower than that in unstimulated cultures (0.66 fold; 95% CI 0.57–0.74 fold, $p < 0.01$). The downregulation of *IL-10* expression in PBMCs of AAP at T_{24} was significantly more pronounced than that in HCs (0.94 fold; 95%CI 0.8–1.34 fold, respectively; $p < 0.01$) (Fig. 1). The upregulation of *IL-13* expression in Dp-stimulated PBMCs of AAPs was seen already at T_6 (10.6 fold; 95%CI 8.3–16.4 fold) being significantly greater than that of HCs (2.2 fold; 95%CI 1.2–6.8 fold; $p < 0.01$) and continued to rise reaching the greatest value at T_{24} , which was again significantly greater than in HCs (52 fold; 95%CI 35.2–70.5 fold and 3.6 fold; 95%CI 2.4–12.7 fold, respectively; $p < 0.01$) (Fig. 1). At T_{144} the expression of *IL-10* and *IL-13* in Dp-stimulated cultures did not differ from those seen in the unstimulated ones (Fig. 1).

Neither in PBMC derived from AAPs nor from HCs significant changes in TGF- β expression in response to Dp stimulation could be demonstrated (not shown).

3.3. Soluble CD163 enhances Dp-induced *IL-10* production by PBMC of AAPs

Soluble CD163 augmented Dp-induced *IL-10* production by PBMCs from AAPs but not from HCs. At T_6 the upregulation of *IL-10* expression by PBMC from AAPs stimulated with Dp in the presence of sCD163 (15.3 fold; 95%CI 10.1–18.7 fold) was significantly greater than that without sCD163 ($p < 0.01$) (Fig. 2). Enhanced expression of *IL-10* could be demonstrated in all Dp-stimulated PBMC of AAPs cultured in the presence of sCD163 as compared to those without sCD163 (Fig. 2). No significant effect of sCD163 on upregulation of *IL-13* expression by Dp could be demonstrated. The amplification of Dp-induced *IL-10* expression by sCD163 was supported by demonstration of enhanced *IL-10* production by PBMCs of AAPs (Fig. 3). The mean *IL-10* concentration in the supernatants of Dp-stimulated PBMCs in the presence of sCD163 (6.17 ± 4.14 pg/ml) was significantly greater than that in cell cultures without sCD163 (3.68 ± 2.06 pg/ml; $p = 0.012$). In the cell cultures derived from each individual AAP greater concentration of *IL-10* was demonstrated in the supernatants of Dp-stimulated PBMC with than without sCD163 (mean increase 64.4%, ranging from 18.4% to 136.8%).

4. Discussion

To the best of our knowledge this is the first study which demonstrates that in AAPs sCD163 can participate in anti-inflammatory response to Dp challenge by enhancing secretion

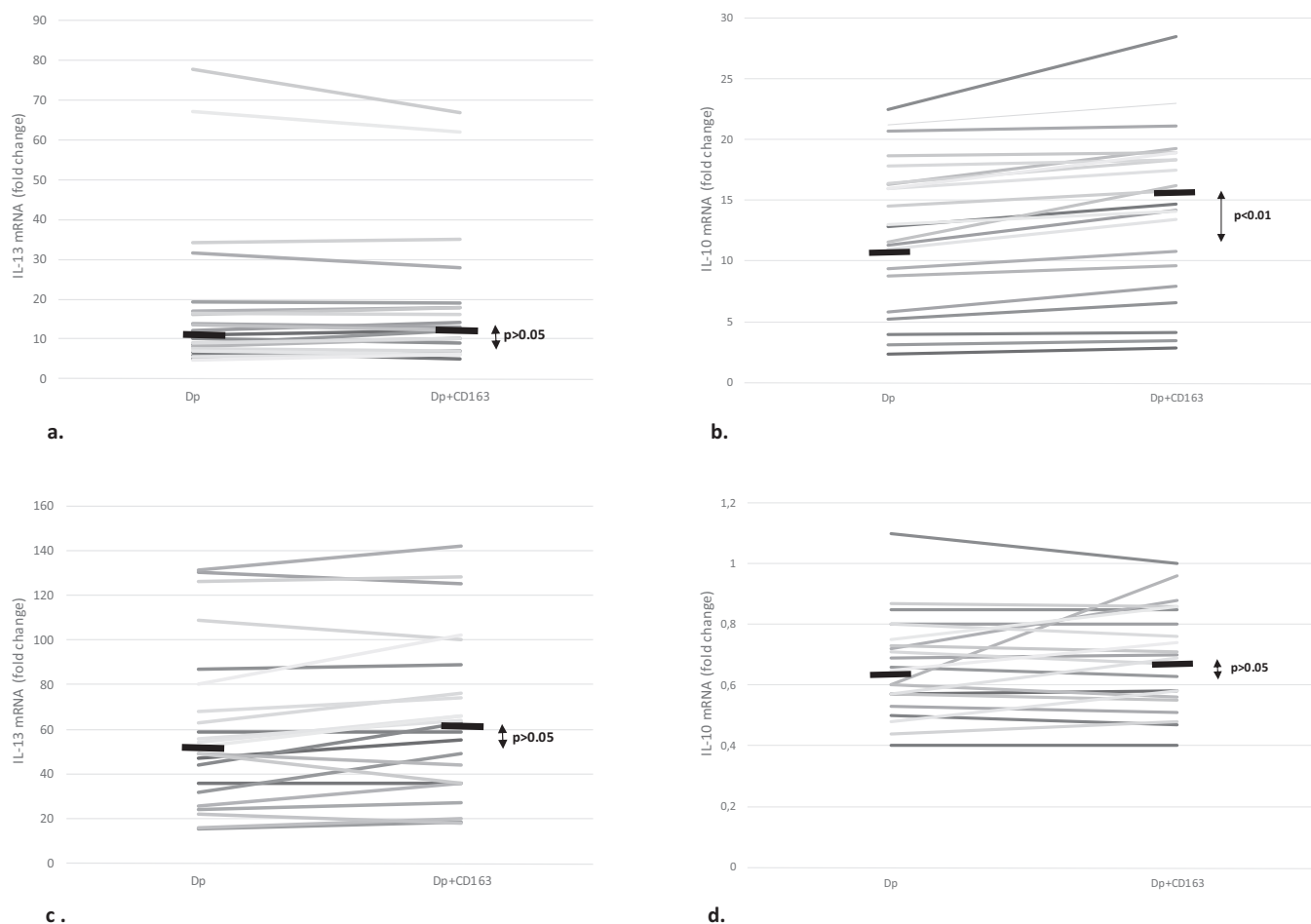


Fig. 2. The effect of sCD163 on Dp-induced expression of *IL-13* and *IL-10* by PBMCs of AAPs after 6 (a and b) and 24 (c and d) hours in culture. Individual lines represent individual patients.

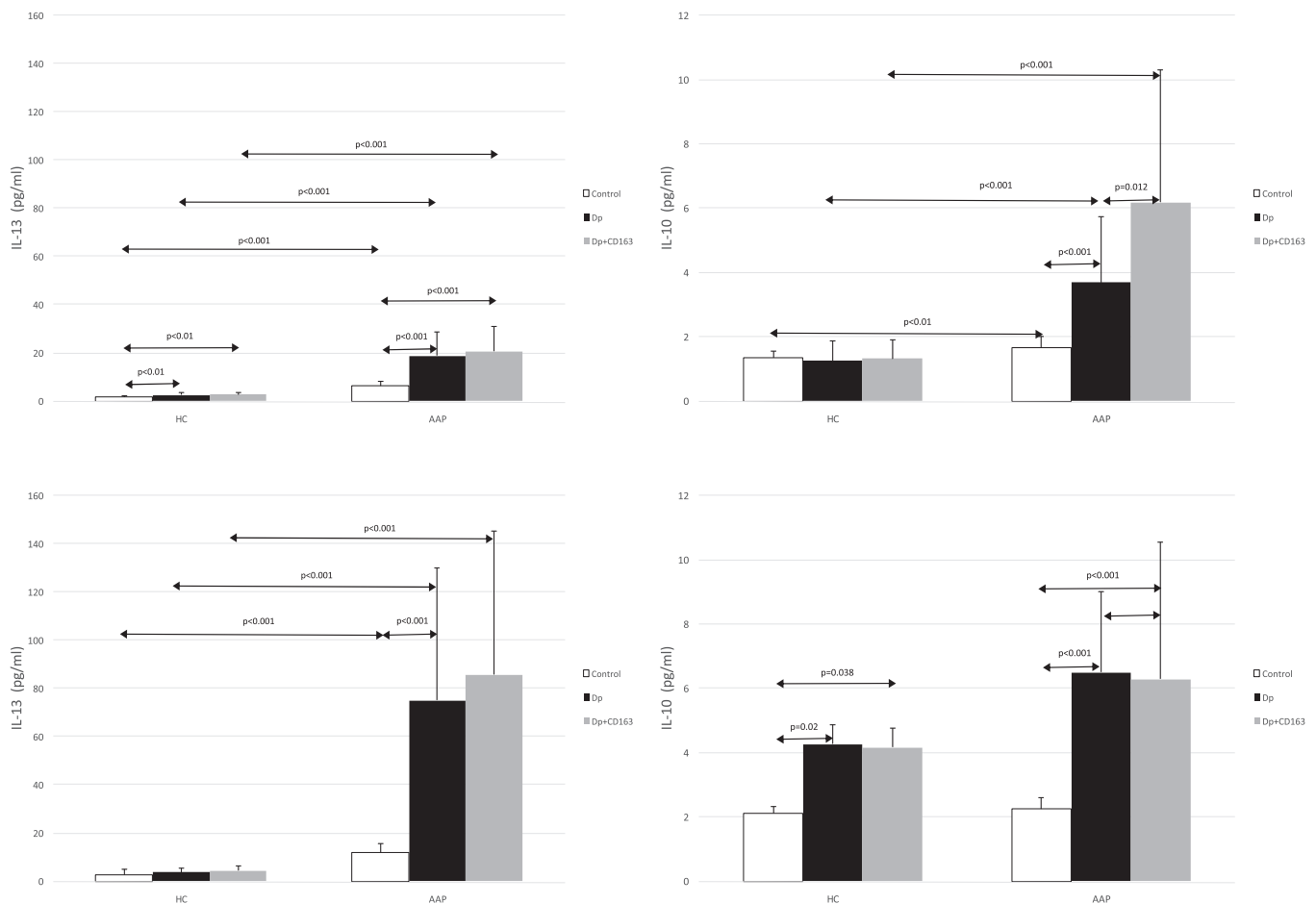


Fig. 3. Concentration of IL-13 and IL-10 in the supernatants of PBMC cultures incubated 24 (upper panel) or 144 (lower panel) hours. The results are expressed as means with standard deviations.

of IL-10 by PBMC of allergic asthmatics stimulated with Dp. The study provides a possible mechanism of anti-inflammatory effects of sCD163 in allergic inflammatory response.

Our results concerning expression of *IL-13* and *IL-10* by allergen stimulated PBMCs of allergic patients are consistent with previously published studies [25–27]. The upregulation of *IL-13* by allergen is robust, but develops slowly with the specific mRNA reaching the greatest values no earlier than 24 h after the stimulation. This is consistent with other studies performed in allergic patients including allergic rhinitis, allergic asthma and atopic dermatitis patients [25,26]. It seems to be allergen specific as we were able to see strong IL-13 production predominantly in PBMCs of AAPs. Using sensitive RT-PCR and high sensitivity ELISA methods we were able to demonstrate marginal up-regulation of *IL-13* mRNA and protein levels in cultures of PBMCs from HCs. The enhanced secretion of IL-13 by Dp stimulated PBMCs from HCs could be demonstrated only at T_{24} but not at T_{144} . These observations are consistent with previously published reports applying different allergens [25–27]. Of particular interest seems to be shown in the current study “early” up-regulation of IL-10 synthesis by PBMCs of AAPs in response to stimulation with Dp. The observation is consistent with a previously published paper evaluating *IL-10* expression by PBMCs derived from ragweed-sensitized allergic rhinitis patients, in which the greatest up-regulation of *IL-10* mRNA was also detected 6 h after stimulation [27]. The rapid induction of *IL-10* by allergen extracts in PBMCs of allergic patients seems to depend on specific, possibly restricted to sensitized patients, recognition of allergen proteins since the

response is observed in allergic patients but not in healthy controls. Moreover, as previously demonstrated ragweed extract can rapidly induce *IL-10* expression in PBMCs derived from ragweed-sensitized allergic rhinitis patients [27]. This supports specific recognition of allergen by PBMCs, which is restricted to allergic patients, as a crucial trigger of rapid *IL-10* expression. Interleukin-10 is a potent anti-inflammatory cytokine and early secretion of IL-10 may inhibit the development of inflammatory response after allergen challenge. Demonstrated in this study up-regulation of *IL-10* expression and secretion by sCD163 provides insights into the anti-inflammatory, homeostatic function of this protein in allergic patients. In fact, an elegant study using a house dust mite-induced experimental allergic airway inflammation model has recently demonstrated that CD163 plays an important homeostatic, anti-inflammatory function in vivo [22]. In mice with silenced *CD163* gene house dust mite-induced allergic airway inflammation is augmented due to enhanced production of pro-inflammatory chemokines [22]. The study provides also an evidence for a direct binding of *D. pteronyssinus* major allergen Der p 1 with mouse CD163 [22]. It is tempting to speculate that possible binding of Der p 1 by human sCD163 may interfere with its specific interaction with inflammatory cells and in this way affect production of anti-inflammatory cytokine IL-10. It remains to be established if and how in humans CD163 affects interaction of *D. pteronyssinus* allergens with human inflammatory cells. Understanding this interaction may have important impact on therapy of allergic diseases including asthma. Corticosteroids which are commonly used in asthma therapy potentially up-regulate

CD163 expression [5–7]. Local administration of corticosteroids to the airways of asthmatic patients resulted in rapid increase of sCD163 in induced sputum of house dust mite-allergic asthma patients [4]. Whether elevated concentration of sCD163 participates in or mediates some anti-inflammatory effects of inhaled corticosteroids remains to be established. The limitation of this study is inclusion of only patients allergic to house dust mites and application of only *D. pteronyssinus* extract for stimulation of PBMCs. We cannot therefore speculated whether the effect of sCD163 is restricted to house dust mite allergens or is functioning in patients sensitized to other allergens. Moreover, it would be interesting to examine the effect of sCD163 on IL-10 production by PBMCs in other inflammatory diseases. In fact elevated production of sCD163 by PBMCs was demonstrated in chronic inflammatory diseases such as systemic sclerosis [28].

5. Conclusions

In summary, in this study we provide evidence for modulating effect of sCD163 on *IL-10* expression by Dp-stimulated PBMCs derived from house dust mite allergic asthma patients. Future studies are warranted to elucidate mechanisms responsible for anti-inflammatory effects of CD163 in allergic inflammation.

Conflict of interests

The authors declare no conflict of interests.

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