

# Bronchial macrophages in asthmatics reveal decreased CD16 expression and substantial levels of receptors for IL-10, but not IL-4 and IL-7

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**Abstract:** The role of different subpopulations of bronchial macrophages (BMs) in asthma pathogenesis has not yet been completely elucidated. In addition, little is known about potential in vivo responsiveness of BMs to pro- and anti-inflammatory cytokines present in the bronchial milieu. We aimed to characterize asthmatic patients' BM subpopulations delineated by common markers of macrophage/monocyte cells, CD16 and CD14, and subsequently to analyze cytokine receptor expression on those subsets. Subjects included eighteen patients with moderate asthma (six steroid-naive and twelve steroid-treated) and ten healthy control subjects. Flow cytometry was used to analyze phenotypical features of BMs including expression of receptors for IL-10, IL-4 and IL-7. Exhaled nitric oxide analysis and induced sputum eosinophil counts were used to assess airway inflammation. BMs from both steroid-naive and steroid-treated asthmatic patients showed significantly decreased expression of CD16, as compared to healthy subjects' BMs. CD16, but not CD14, expression inversely correlated with exhaled nitric oxide levels and sputum eosinophilia. Short-term administration of inhaled corticosteroids (ICS) in steroid-naive asthmatic patients led to significant reduction of CD16 expression and enhancement of CD14 expression. Next, we analyzed the expression of receptors for IL-10, IL-4 and IL-7 on the surface of BM subpopulations characterized by different levels of CD14 and CD16 expression. We observed substantial levels of IL-10R on the surface of BMs collected from asthmatic and healthy subjects. Interestingly, IL-10R was found mostly on those macrophages that co-expressed CD14. In contrast, independently on co-expression of CD14, the levels of IL-4R and IL-7R on BMs were low in both asthmatic and healthy subjects. The results suggest that different BM subsets may be differentially involved in regulating the inflammatory response in allergic asthma.

**Key words:** CD16 - IL-10R - IL-4R - IL-7R - Bronchial macrophages - Nitric oxide - Asthma

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## Introduction

The airway macrophages (AMs) represent a heterogeneous group of cells that actively participate in the regulation of immune and inflammatory activities in lung diseases [1-4]. The role of AMs in the pathogenesis of allergic airway inflammation is complex and is often determined by their activated phenotype [5]. To date, the phenotypic features of AMs have been characterized in healthy and asthmatic subjects [6-10]. However, the majority of reports were based on the analysis

of cells collected from bronchoalveolar lavage (BAL) fluid, thus representing predominantly lower airways [11]. Although this method provides a valuable tool for assessment of airway cells, safety issues and access to expert resources may limit its wide use in daily practice [12]. In contrast, sputum induction provides an easy, repeatable and non-invasive tool for collection of large numbers of bronchial macrophages (BMs) together with eosinophils that serve as common markers of allergic inflammation [13]. BMs constitute a large population of AMs involved both in regulation of innate immunity processes and allergic inflammation [14]. Sputum/bronchial phagocytes (among others macrophages) express an inflammatory phenotype even in healthy individuals and are functionally more active than macrophages from lower airways [15]. In another report, the same group described phagocytic

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**Table 1.** Subject characteristics.

Subject	Age	Sex	Atopy	Asthma history	ICS use	LABA use	% predicted FEV1
Asthmatic ICS (-)	41.3 (SD ± 8.4)	F-3 M-3	Yes	8.6 yrs (6-10 yrs)	No	Yes	74.6 % (SD ± 19.7)
Asthmatic ICS (+)	46.7 (SD ± 7.7)	F-8 M-4	Yes	9.8 yrs (7-18 yrs)	600-800 µg of BUD	Yes	84.3 % (SD ± 11.3)
Control	38.9 (SD ± 6.2)	F-6 M-4	No	No	No	No	108.1 % (SD ± 8.3)

Values are means ± standard deviation (SD). M-male, F-female. Asthmatic ICS (-) - asthmatic patients not receiving ICS. Asthmatic ICS (+) - asthmatic patients treated with ICS. BUD - budesonide. LABA - long-acting β-agonists. FEV1- forced expiratory volume in 1s

dysfunction of BMs in asthmatic patients [16]. Thus far, only few groups attempted to characterize and enumerate BM subsets in asthmatic patients and normal atopic and non-atopic subjects [17,18]. However, less is known about phenotypical features of different subpopulations of BMs. This is mostly due to the fact that, in general, the majority of reports on AMs were based on the application of one- or two-color flow cytometry or immunocytochemistry. In this study, using a three-color flow cytometric approach, we present the differences between asthmatic and healthy subjects in phenotype of BMs, also with regard to expression of receptors for pro- and anti-inflammatory cytokines, namely IL-4, IL-7 and IL-10. The counteracting roles of IL-4 and IL-10 in modulating allergic inflammation have been widely described [19-21]. IL-7 has been postulated a "master regulator" of lymphocyte T-dependent immune responses; however, it was also shown to exert profound effects on populations of tissue macrophages [22-25]. Recently, polymorphisms in genes encoding IL-7R were associated with asthma symptoms, thus suggesting the role for IL-7 and its receptor in asthma pathogenesis [26]. Moreover, in a recent report we demonstrated that IL-7R expression is decreased in lungs of SIV-infected non-human primates, indicating that IL-7R may be a marker of ongoing lung-associated inflammation [27]. Altogether, based on the above information, we hypothesized that the levels of receptors for IL-10, IL-4 and IL-7 might be important in determining the susceptibility of BMs to immunomodulatory signals.

In this study we attempted to correlate flow cytometric phenotypic data regarding BMs with analyzed on daily basis non-invasive markers of asthma severity, including exhaled nitric oxide. In addition, we analysed the effects of short-term inhaled corticosteroid (ICS) treatment on the phenotype of BMs in steroid-naive asthmatic patients. The data obtained suggest, among others, an interesting correlation between CD16 expression and eosinophilic airway inflammation and between CD14 expression and IL-10R expression on BMs.

## Materials and methods

**Subjects.** Subject characteristics are shown in Table 1. Severity of asthma was classified according to GINA (Global Initiative for Asthma) criteria [28]. Six patients with moderate persistent asthma who were receiving treatment with only the inhaled long-acting beta-adrenergic agonists were recruited as members of the "steroid-naive" group. Those patients demonstrated >15% improvement in FEV1 (forced expiratory volume in 1s) following 200µg of salbutamol. Twelve patients with moderate persistent asthma treated with a combination of medium doses of inhaled glucocorticosteroids and long-acting beta-adrenergic agonists were recruited to the "steroid-treated" group. Those patients were previously diagnosed in The Department of Allergology of Medical University Hospital in Bialystok and subsequently treated for asthma for a period of 7-18 years. All patients from both asthmatic groups were atopic as defined by at least two positive skin prick tests to common allergens. Current smokers and ex-smokers were excluded from the study.

In order to compare the bronchial macrophages between asthmatic and nonasthmatic patients, we studied ten healthy nonatopic nonsmoking volunteers who gave no history of asthma or of any respiratory disease and who had normal lung function. The study protocol was approved by the Bialystok Local Ethics Committee (R-I-003/152/2005).

**Induced sputum.** Sputum was induced by the inhalation of hypertonic saline as described elsewhere [29]. Three 7-min. inhalation periods of 3, 4 and 5% hypertonic saline were administered following baseline spirometry. All subjects were premedicated with 200µg of inhaled salbutamol before saline inhalation. After each inhalation period, an FEV1 value was measured for safety reasons and the subjects were asked to blow their nose and rinse their mouth with water, and to perform a "chesty-type" cough. The sputum was expectorated into a sterile container that was placed on ice throughout the procedure.

Sputum was selected from saliva and processed immediately after the collection as described by Pizzichini *et al* [29]. In brief, sputum was weighed and incubated for 15 min at room temperature with a volume of 0.1% DTT (dithio-threitol) equal to four times the weight (mg), and subsequently washed with four times the plug weight in Dulbecco's PBS. The suspension was filtered through a 48 µm-pore mesh filter and used for total cell count and cell viability with the use of trypan blue. Visually identifiable squamous epithelial cells were not counted or included in the total cell count. Differential cell counts were performed on a minimum of 400 nonsquamous cells.

**Immunostaining.** Aliquots of 100 µl of sputum cell suspension ( $1 \times 10^6$  cells/ml) were incubated with 20 µl of monoclonal anti-

bodies: anti-CD14 FITC or anti-CD14 PE (BD PharMingen, Belgium), anti-CD16 PE-Cy5 or anti-CD16 FITC (BD PharMingen, Belgium), anti-CD210 PE (IL-10R, BD PharMingen, Belgium), anti-CD124 PE (IL-4R, BD PharMingen, Belgium), and anti-CD127 PE (IL-7R, Immunotech, France). The cells were stained with fluorochrome-conjugated mAbs for 30 min at room temperature, washed twice with phosphate-buffered saline, and fixed with 2% paraformaldehyde.

**Flow cytometric analysis.** Flow cytometry was performed as described previously [30,31]. A FACSCalibur cytometer (Becton Dickinson Immunocytometry Systems) was used to determine the surface intensity of various antigens on sputum macrophages. In order to ensure the reproducibility of the data generated, the settings and calibration of the instrument fluorescence detectors were monitored and optimized on each day of analysis according to manufacturer's recommendations, using CaliBRITE beads (BDIS). The system linearity was evaluated using Sphero Rainbow Beads (Spherotech, Libertyville, IL). The coefficient of variation (CV) of all control beads used was less than 3% for scatter and fluorescence. Flow cytometry data were collected in list mode and analyzed using CellQuest software (BDIS). First, the bronchial macrophage population was gated by a combination of forward and side scatter. The gated cells were found positive for CD68 (data not shown). To assess the expression of CD14, CD16, CD124, CD127, and CD210, the gate was initially set with corresponding isotype and FMO (fluorescence-minus-one) controls. In accordance with opinion of Roederer *M et al.*, the data are presented as the geometric mean fluorescence intensity of bronchial macrophages coexpressing the above antigens [32]. An example of raw data for negative and positive control samples, and, CD14 FITC and CD16 PE-Cy5 expression is shown on Fig. 1A and, the raw data for IL-10R expression are shown on Fig. 3A.

**Exhaled nitric oxide measurements.** The concentration of nitric oxide in the exhaled air was assessed with the use of chemiluminescence analyzer NOA™ 280i (Sievers, USA) according to American Thoracic Society recommendations as described previously [33,34]. In brief, the analyzer was fitted with a biofeedback display unit to provide visual guidance for the subject to maintain the pressure and exhalation flow within a certain range. Subjects exhaled slowly from total lung capacity over 30 s with an exhalation flow of 50 ml/s. The mean of three separate measurements was used for analysis.

**Statistical analysis.** We used Kruskal-Wallis ANOVA and Mann-Whitney nonparametric tests to analyse statistically significant differences among groups. Nonparametric Wilcoxon test was used to analyse the pre- and after-treatment values. The correlations were examined by Spearman rank correlation coefficient. In all tests, statistically significant results were identified by a p value of <0.05.

## Results

### *CD16 expression is decreased in BMs of asthmatic patients*

The use of a combination of CD14 and CD16 monoclonal antibodies revealed dramatically different BM phenotype profiles between both untreated and treated asthmatics and healthy subjects. First, based on unstained, isotype and FMO controls and different densities of CD14 and CD16 staining observed in healthy subjects, we delineated four distinct BM subpopulations (Fig. 1A) Such phenotype was consistent in all healthy controls, thus we set the addi-

**Table 2.** Percentage distribution of bronchial macrophages among BMs with different intensities of CD14 and CD16 expression.

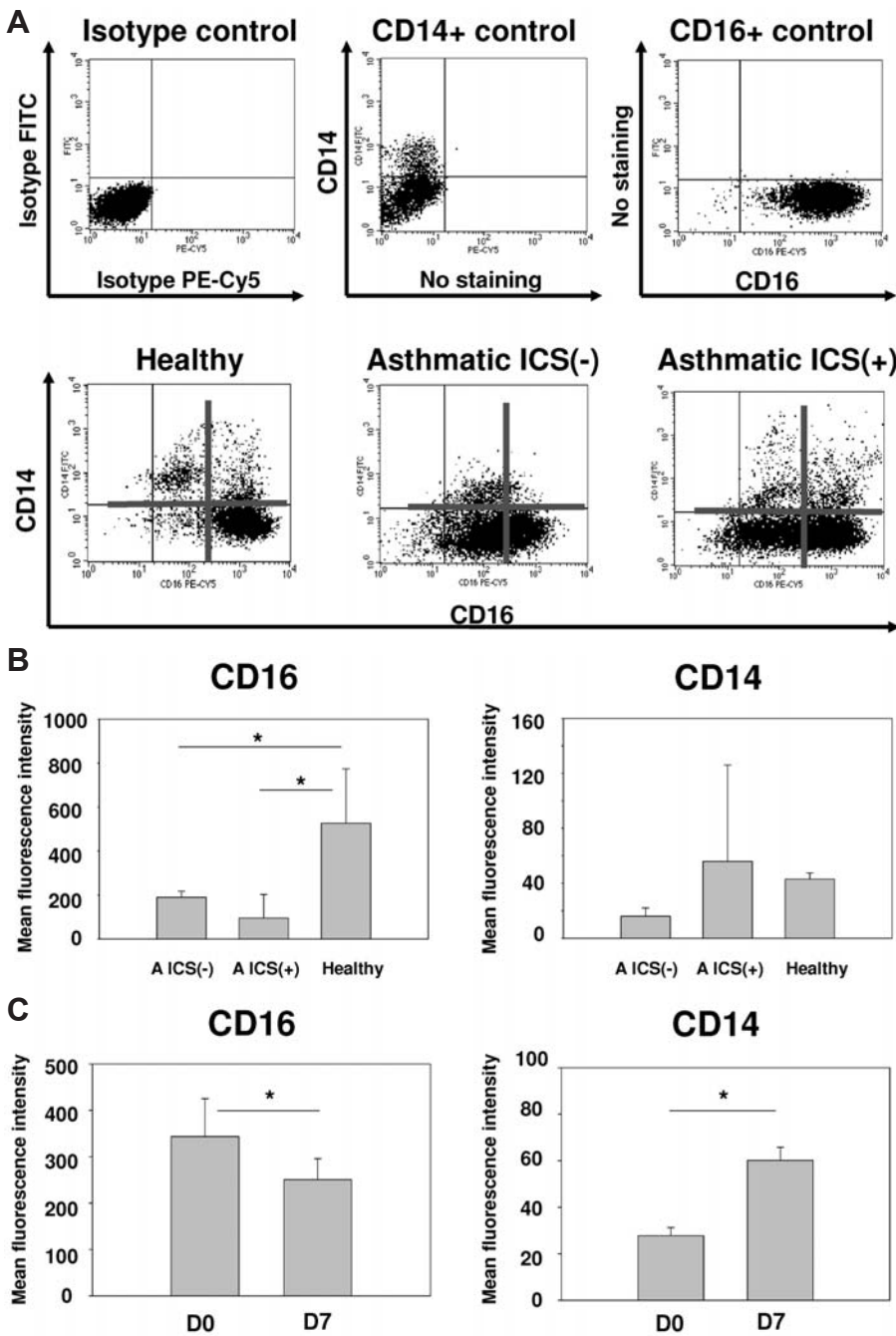
BM phenotype	Asthmatic ICS (-)	Asthmatic ICS (+)	Control
CD14low CD16dim	52.6% (38.4-61.7)	60.4% (47.6-69.5)	6.8% (4.5-8.8)
CD14high CD16dim	8.3% (5.2-10.4)	6.3% (4.3-9.7)	10.1% (7.6-12.3)
CD14high CD16high	1.3% (0.8-2.1)	4.3% (3.1-6.5)	4.8% (3.3-5.6)
CD14low CD16high	39.4% (30.5-44.9)	28.3% (23.1-34.8)	78.4% (68.2-86.5)

Phenotypic division of BMs refers to varying levels of CD14 and CD16 expression as depicted on Fig.1A with the use of grey quadrant lines. Values are presented as medians with 25th-75th percentiles..

tional quadrant markers (thick grey lines) separating those subpopulations and kept them throughout the study for further comparative analysis of BM phenotype in asthmatic patients (Fig. 1A, bottom panel). In brief, we found that about 80% of BMs in healthy subjects is characterized by the CD14low CD16high phenotype. In contrast to healthy controls, asthmatics' BMs revealed mostly CD14low CD16dim and CD14low CD16 high phenotype. Detailed percentage distributions of BMs with different intensities of CD14 and CD16 expression are presented in Table 2. Notably, in asthmatics, there was no clear distinction between CD14low CD16dim and CD14low CD16high BMs. Thus, in order to more precisely quantify the phenotypic differences among subjects, we analyzed total antigen densities for both CD14 and CD16 (Fig. 1B). BMs from steroid-naive and steroid-treated asthmatics exhibited significantly decreased CD16 expression as compared with healthy subjects ( $p < 0.05$  for both groups). In contrast, CD14 presented with a different pattern of expression. Although we did not find statistically significant differences among groups, steroid-treated asthmatics tended to express more CD14 than steroid-naive patients, and, to a lesser extent, healthy subjects.

### *Short-term ICS administration results in decrease of CD16 expression and enhancement of CD14 expression*

To investigate the potential role of CS treatment in modulation of CD14 and CD16 expression on the surface of BMs, in four of six steroid-naive asthmatics the follow-up flow-cytometric analysis was performed after 7 days of treatment with 800 µg of inhaled budesonide. As depicted in Fig. 1C, short-term ICS treatment exerted opposing effects on CD14 and CD16 BM expression. BMs tested showed a decrease in expression of CD16 and enhancement of CD14 expression ( $p < 0.05$ , for both molecules).



**Fig. 1.** The analysis of CD14 and CD16 expression in BMs of healthy and asthmatic subjects. **A.** Flow cytometric density plots characteristic for healthy and asthmatic patients. Standard quadrants (thin black lines) were created based on isotype and CD14+ and CD16+ FMO controls from healthy subjects (top panel). Additional thick grey quadrant lines were drawn to delineate four distinct BM subpopulations based on the FMO controls and the pattern of expression of CD14 and CD16 observed in healthy subjects (bottom left density plot). Thus CD14<sup>low</sup> CD16<sup>dim</sup> BMs are in the lower left quadrant, CD14<sup>high</sup> CD16<sup>dim</sup> in the upper left quadrant, CD14<sup>high</sup> CD16<sup>high</sup> in the upper right quadrant, and CD14<sup>low</sup> CD16<sup>high</sup> in the lower right quadrant. **B.** The summary of analyses of CD16 and CD14 expression by BMs in asthmatic and healthy subjects. The results are for the geometric mean fluorescence intensity (MFI) of the CD16+ (left column) and CD14+ (right column) BMs, reported as the median with 25th-75th percentile. Statistically significant differences between groups are indicated with asterisks ( $p < 0.05$ ). Asthmatic ICS (-) - asthmatic patients not receiving ICS. Asthmatic ICS (+) - asthmatic patients treated with ICS. **C.** The effects of 7-day administration of 800  $\mu\text{g}$  of inhaled budesonide in steroid-naïve asthmatic patients on the levels of CD16 and CD14 on the surface of BMs. The results are for the MFI of the CD16+ (left column) and CD14+ (right column) BMs, reported as the median with 25th-75th percentile ( $n=4$ ). Statistically significant differences between groups are indicated with asterisks ( $p < 0.05$ ).

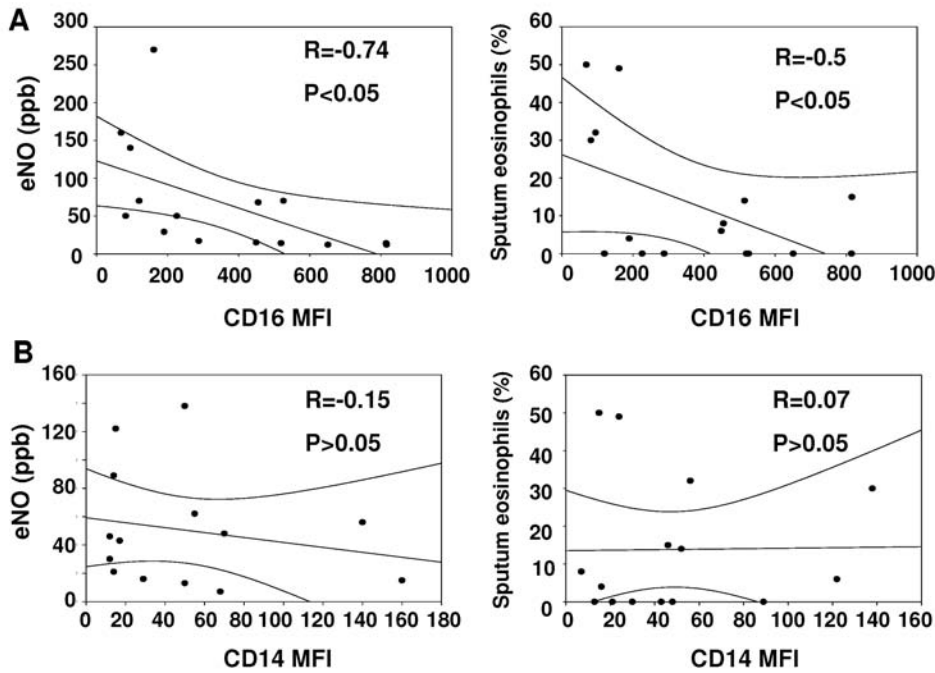
### ***CD16, but not CD14, expression in BMs inversely correlates with exhaled nitric oxide and sputum eosinophilia***

As both CD16 and CD14 molecules appeared to be modulated in asthmatic patients, we investigated whether correlations between their expression and markers of asthma severity exist. In fact, significant inverse correlations were observed between CD16 expression and exhaled nitric oxide (eNO) ( $r = -0.74$ ,  $p < 0.05$ ), and sputum eosinophilia ( $r = -0.5$ ,  $p < 0.05$ , Fig. 2A). In contrast, no significant correlations were found when similar analyses were performed for CD14 (Fig.

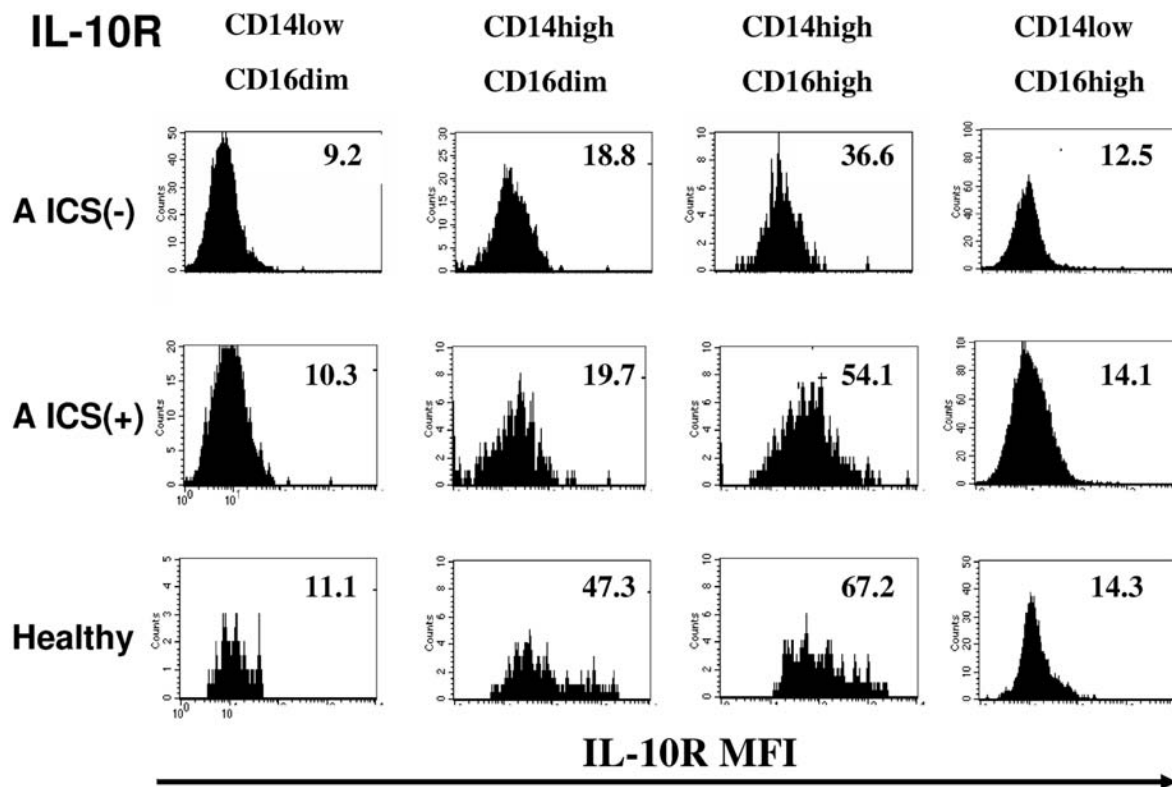
2B). Congruently with the observations made by Jatakanon *et al.* [35], exhaled nitric oxide and sputum eosinophilia in studied subjects correlated closely with each other ( $r = 0.58$ ,  $p < 0.05$ ).

### ***BMs express substantial levels of IL-10R that positively correlate with CD14 expression***

Next we applied three-color flow cytometry to analyse the expression of IL-10R on the surface of BMs with different intensities of CD14 and CD16 expression. Interestingly, in all three groups of patients we



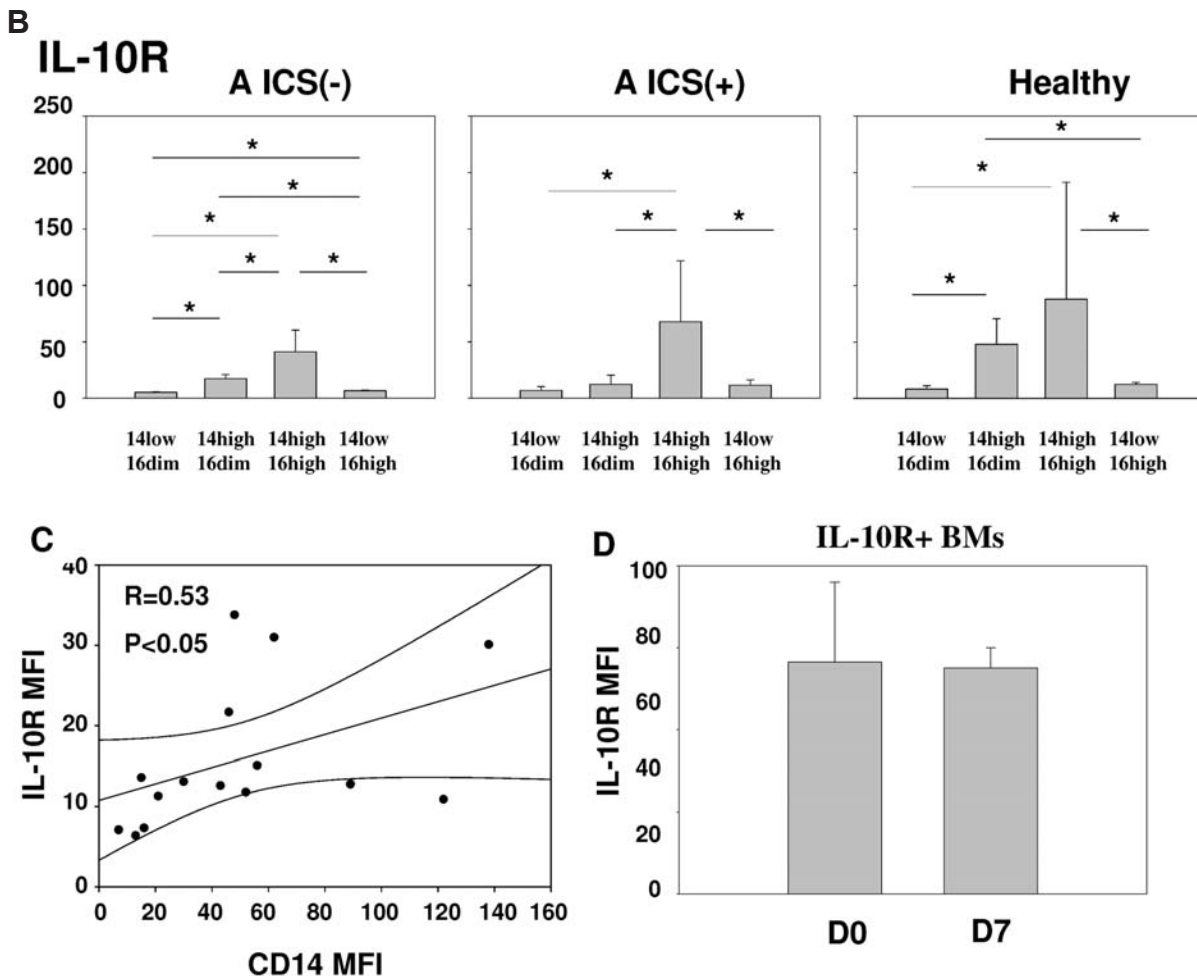
**Fig. 2.** A. Linear regression analysis of BM CD16 expression and exhaled nitric oxide (eNO) levels (left panel) and sputum eosinophils (%) (right panel). A significant ( $p < 0.05$ ) negative correlation was observed between CD16 expression and eNO levels ( $r = -0.74$ ) and percent of sputum eosinophils ( $r = -0.5$ ). B. Similar analysis performed for CD14 did not reveal statistically significant correlations:  $r = -0.15$  for eNO (left panel,  $p > 0.05$ ), and,  $r = 0.07$  for sputum eosinophils (%) (right panel,  $p > 0.05$ ).



**Fig. 3A.** Flow cytometric histograms representing the levels of IL-10R expression in BMs with different intensities of CD14 and CD16 staining in representative samples of asthmatic and healthy subjects. Values refer to MFI of IL-10R expression by marked BMs.

observed a similar profile of IL-10R distribution between BM subpopulations. As depicted in Fig. 3A, and summarized in Fig. 3B, IL-10R was found on the surface of CD16<sup>dim</sup> CD14<sup>high</sup> and CD16<sup>high</sup>

CD14<sup>high</sup> BMs at two- to ten-fold higher levels than on CD16<sup>dim</sup> CD14<sup>low</sup> and CD16<sup>high</sup> CD14<sup>low</sup> BMs. Moreover, we found a positive correlation between CD14 and IL-10R expression ( $r = 0.53$ ,  $p < 0.05$ , Fig. 3C).



**Fig. 3B-D.** **B.** The summary of analyses of IL-10R expression by BMs in asthmatic and healthy subjects. The results are for the MFI of the IL-10R+ BMs, reported as the median with 25th-75th percentile. Statistically significant differences between groups are indicated with asterisks ( $p<0.05$ ). **C.** Linear regression analysis of BM IL-10R expression and CD16 expression. A significant ( $p<0.05$ ) positive correlation was observed between IL-10R expression and CD16 expression ( $r=0.53$ ). **D.** The effects of 7-day administration of 800  $\mu\text{g}$  of budesonide in steroid-naïve asthmatic patients on the levels of IL-10R on the surface of BMs. The results are for the MFI of the IL-10R+ BMs, reported as median with 25th-75th percentile ( $n=4$ ).

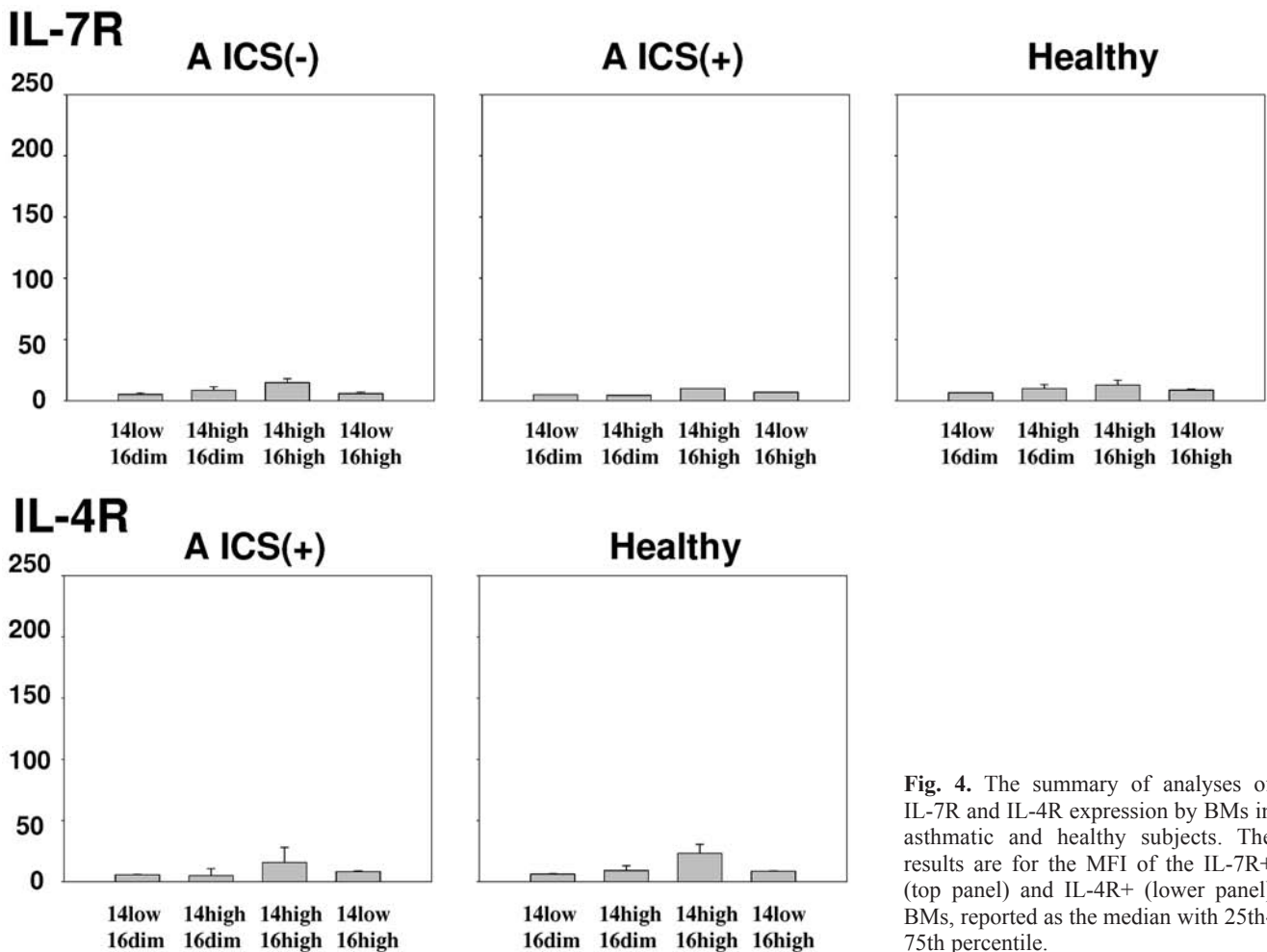
Comparative analysis of IL-10R expression on corresponding BM subpopulations in healthy and asthmatic patients did not reveal statistically significant differences among groups. However, we observed that predominantly CD14+ BMs of healthy individuals tended to express more IL-10R as compared with both asthmatic groups. Finally, we investigated the effects of short-term ICS treatment on modulation of macrophage IL-10R expression in steroid-naïve asthmatics. Surprisingly, we observed varying changes in IL-10R expression, which resulted in a lack of significant difference between pre- and after-treatment values for total BM population as well as single BM subsets (Fig. 3D and data not shown). Moreover, we did not find statistically significant correlations between levels of IL-10R and eNO and sputum eosinophilia ( $r=0.15$ ,  $p>0.05$ , and,  $r=0.17$ ,  $p>0.05$ , respectively).

#### Low levels of IL-7R and IL-4R on BMs

Three-color flow cytometry analysis revealed that BMs of all studied subjects expressed both IL-7R and IL-4R at significantly lower levels than IL-10R. Interestingly, in contrast to IL-10R, IL-7R and IL-4R expression was equally distributed among all BM subpopulations (Fig. 4). Moreover, we did not observe any differences in IL-7R and IL-4R among asthmatic and healthy individuals. Finally, the effects of short-term ICS treatment on the levels of IL-7R and IL-4R were negligible (data not shown).

#### Discussion

In our study we provided novel phenotypic characteristic of BMs with regard to cytokine receptor expression. Notably, we correlated data concerning BMs with



**Fig. 4.** The summary of analyses of IL-7R and IL-4R expression by BMs in asthmatic and healthy subjects. The results are for the MFI of the IL-7R+ (top panel) and IL-4R+ (lower panel) BMs, reported as the median with 25th-75th percentile.

non-invasive markers of allergic inflammation. Our results show that eosinophilic inflammation in asthmatic patients is closely associated with decreased CD16 expression on BMs. CD16, together with CD64, participates in phagocytosis of opsonized particles or of immune complexes; thus, its decreased expression in asthmatics suggests that allergic inflammation may disturb phagocytic activity of BMs. The results presented here in moderate asthmatics supplement the findings reported by Alexis *et al.* in mild asthmatics, *i.e.*, dysfunctional phagocytosis of IgG-opsonized yeast and decreased CD64 expression [16].

We demonstrated that ICS treatment resulted in significant reduction of CD16 expression on BMs. Our results are in line with reports of other researchers investigating the effects of CS treatment on peripheral blood monocytes. Fingerle-Rowson *et al.* and Dayyani F *et al.* reported a significant decrease in numbers of CD16+ monocytes in patients treated for multiple sclerosis with high doses of glucocorticoid therapy [36,37].

As we have shown in our study, CD14+ expression on BMs positively correlates with expression of the receptor for IL-10. Surprisingly, short-term ICS

administration did not result in unambiguous enhancement of IL-10R expression despite enhancement of CD14 expression. Similarly, we did not find significantly higher IL-10R levels on BMs of asthmatic patients that received long-term ICS therapy. Thus, it is possible that the effects of ICS on IL-10R expression might be influenced by other cells and/or inflammatory mediators accounting for chronic persistence of allergic airway inflammation.

IL-10 has numerous anti-inflammatory effects and its action is mediated by IL-10R [17,38]. Lim *et al.* reported detectable IL-10R expression in alveolar macrophages but not epithelial cells collected from BAL [21]. Those authors did not find differences in macrophage IL-10R expression between asthmatic and healthy subjects. Our results suggest that healthy subjects' BMs (especially CD14+) tend to express more IL-10R as compared to asthmatic subjects. Further studies are warranted to investigate whether BMs of asthmatic patients may indeed have less pronounced functional response to IL-10. Our work extends the observations of Lim *et al.* to BMs as we have shown that those cells express substantial levels of receptors for IL-10. This

could be of clinical importance as BMs constitute a large population of airway cells that could benefit from either therapy stimulating local IL-10 production or even potential IL-10 administration [39-41].

Differential expression of IL-10R by CD14+ and CD14-negative BMs suggests that not all BMs play identical roles in regulation of allergic inflammation. Whilst CD14+ BMs constitute only a small percentage of the total population, they seem to be most susceptible to IL-10-mediated signals. Detailed functional studies are needed to investigate whether BMs characterized by monocyte-associated phenotype (CD14+) may play a more significant role in immunoregulation of allergic inflammation as compared with the majority of CD14-negative BMs.

Detection of low levels of IL-4R and IL-7R on the surface of BMs is in line with similar results obtained in peripheral blood monocytes [42], and our own observations]. Thus, it cannot be solely associated with presumable laboratory artifacts caused by, for example, isolation procedures [30]. In fact, many studies show that the use of DTT in sputum preparation may influence the obtained results [43,44]. On the other hand, detection of low levels of IL-4R and IL-7R, despite the use of a technical protocol identical to that for IL-10R analysis, clearly indicates the differential expression of those three receptors. The functional relevance of our findings regarding the expression of IL-4R and IL-7R on BMs remains to be elucidated in further in vitro studies.

In summary, our data demonstrate that BM phenotype is affected by both airway inflammation process and ICS treatment. Moreover, we suggest that BMs are capable of responding to immunomodulatory signals, mainly those mediated by IL-10.

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