



Phenotypic analysis of alveolar macrophages and lymphocytes following allergen inhalation by atopic subjects with mild asthma[☆]

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KEYWORDS

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Summary

Background: The airway inflammation associated with allergic asthma is initiated through a complex interaction of antigen-presenting cells (APC) and T lymphocytes resulting in the release of a cascade of cytokines regulating the progress of the allergic inflammatory response. In the present study the state of alveolar macrophage (AM) and T cell activation was investigated following induction of allergic airway inflammation in individuals with atopic asthma.

Methods: Eleven individuals with mild, atopic asthma received cumulated allergen inhalations. Before and one day after challenge, bronchoalveolar lavage (BAL) was performed, and peripheral blood samples obtained. Ten healthy individuals served as controls. The expression of cell surface markers by BAL fluid AMs and T cells, and by blood T cells, was investigated by flow cytometry.

Results: All patients developed early asthmatic reactions (EAR) with increased numbers of eosinophils and mast cells in BAL fluid following allergen challenge. After allergen challenge, patients had relatively fewer pulmonary CD4⁺ T cells expressing CD69 and HLA class II and also relatively fewer pulmonary CD8⁺ T cells expressing HLA class II, compared to before challenge. An increased quantitative expression of CD14 and CD86 was seen within the AM population following allergen challenge.

Conclusion: The results indicate a recruitment of non-activated, immature macrophages and CD4⁺ T cells to the airways as well as an altered phenotype pattern within the AM population following induction of allergic airway inflammation by allergen inhalation challenge in asthma.

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Introduction

Allergic asthma is associated with an airway inflammation characterized by a tissue infiltration of eosinophils, monocytes and lymphocytes. The allergic inflammatory response is initiated through a complex interaction of antigen-presenting cells (APC) and T lymphocytes, resulting in a predominance of T helper 2 (Th2) cells secreting key cytokines such as IL-4, IL-5 and IL-13, which regulate the allergic inflammatory response.¹ Alveolar macrophages (AMs) may influence the Th1/Th2 balance in the asthmatic lung, since it has been shown that under certain conditions distinct subsets of macrophages may skew the cytokine profile of T cells in either the Th1 or Th2 direction. Regulatory cytokines, such as IL-10 and TGF β can also be produced by AMs. The role of AMs in asthma was recently reviewed in Peters-Golden.² In the healthy lung, AMs are generally considered to exert a downregulatory effect on acquired immune responses, exemplified by the enhanced antigen presentation by dendritic cells following AM depletion.³ In a rat model of allergen-induced asthma, in vivo depletion and adoptive transfer of AMs between resistant and susceptible strains demonstrated that AMs suppressed airway hyperresponsiveness.⁴ However, under certain conditions there may be a failure of AM suppressive mechanisms or even a switch to a co-stimulatory activity that could contribute to the airway inflammation, characterized by T cell activation, Th2 cytokine secretion and eosinophilia seen in allergic asthma. AMs of asthmatic individuals express several activation markers to a higher degree than in healthy controls (HC).^{5–7} One study showed that this expression is further enhanced by allergen challenge and that levels of several AM markers correlated with airway sensitivity to metacholine.⁸ The expression of pro-inflammatory mediators also differ in AMs from asthmatics compared to controls,^{9–11} and studies by one group have shown that AMs from atopic asthmatics have a higher capacity to induce IL-5 production in CD4⁺ T cells than do AMs from atopic individuals without asthma.^{12,13} A Th2 stimulatory role for AMs has also been demonstrated in idiopathic pulmonary fibrosis.¹⁴ However, more studies are clearly needed to better appreciate the role of AMs in allergic asthma.

To investigate the AM/T cell expression of surface markers related to activation and co-stimulation during in vivo allergen exposure, we analyzed by flow cytometry the phenotypic pattern of cells recruited by bronchoalveolar lavage (BAL), before and after birch or grass allergen inhalation by individuals with mild atopic asthma.

Material and methods

Subjects

Eleven patients with mild asthma and radioallergosorbent test (RAST) and skin prick test-verified allergy to birch pollen ($n = 9$) or grass pollen ($n = 2$) were recruited for the study. All patients were non-smokers and had previously documented asthmatic reactions following allergen bronchoprovocation. The study population included two males and nine females, mean age 29 (range 25–32). The patients were all in a stable phase of disease with FEV_{1.0} values ranging from 82.0% to 106.0% of predicted and received medication with β -agonists as needed only. Ten healthy non-smoking controls, five males and five females, mean age 30 (range 19–50) were included in the study. All patients and subjects had normal chest radiographies and were without signs of infection at the time of the study; part of the results from the HC were also used in another study.¹⁵ Written informed consent was obtained and the study was approved by the local ethics committee.

Study design

The study was performed outside the pollen season. Allergen extracts used (Aquagen, ALK, Copenhagen, Denmark) were standardized and freeze dried. Peripheral blood and BAL samples were obtained 2–3 weeks before and 1 day after the allergen inhalation challenge. All bronchoprovocations were performed using a dosimeter-controlled jet nebulizer (Spira Elektro 2, Respiratory Care Center, Hameenlinna, Finland) as previously described.¹⁶ In brief, the allergen challenge started with inhalation of diluent. Provided that FEV_{1.0} did not change more than 10%, allergen was inhaled every 15 min with half-log increments of the cumulated dose, until FEV_{1.0} dropped 20% or more from the post-diluent baseline value. Late asthmatic reactions (LAR) were monitored by measurements of peak expiratory flow (PEF) rates during 24 h following allergen challenge. Bronchial provocations with methacholine were performed in a similar manner one day before and two days after the allergen inhalation challenge. The BAL fluid was collected from HC without prior allergen or methacholine challenge.

BAL and handling of cells

Bronchoscopy with BAL was performed as previously described.¹⁷ Briefly, BAL fluid was retrieved

by instilling five aliquots of 50 ml sterile phosphate buffered saline (PBS) solution at 37 °C in a middle lobe bronchus. After each instillation the fluid was gently aspirated and collected in a siliconized bottle kept on ice. The BAL fluid was strained through a double layer of Dacron nets (Millipore, Bedford, Ireland), centrifuged at 400g for 10 min at 4 °C and the cells were resuspended in RPMI 1640 (Sigma Aldrich Co., St. Louis, MO, USA). The BAL fluid cells were counted in a Bürker chamber and cell viability (mean = 85%) was determined by Trypan blue exclusion. For differential cell counts, cytopspins were prepared by cytocentrifugation at 20g for 3 min and stained in May–Grünwald Giemsa. Peripheral blood lymphocytes (PBL) were separated from heparinized peripheral blood by Ficoll–Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, washed twice and diluted in RPMI 1640.

Immunostaining and flow cytometry

Immunolabeling of AMs was performed using primary non-conjugated monoclonal antibodies (listed in Table 1) followed by a secondary phycoerythrin (RPE)-conjugated antiserum. Antibodies specific for CD58 and CD83 with isotype-matched controls were obtained from Serotec (Oxford, UK). CD1a, CD14, CD16, CD54, CD86 and HLA class I and class II specific antibodies with isotype-matched controls were obtained from DAKO (Glostrup, Denmark). Phenotypic characterization of lymphocytes was performed using FITC-labeled monoclonal antibodies specific for HLA class II (Dako) and CD69 (Becton Dickinson, Mountain View, CA, USA).

Monoclonal RPE-Cy5 conjugated CD4 antibodies, RPE conjugated CD8 antibodies and FITC conjugated CD3 antibodies were obtained from Dako. Triple staining of cells was performed to determine the expression of activation markers by CD4+ and CD8+ cells, respectively, and to assess the percentage of CD3+ cells that expressed CD4 and CD8. After antiserum incubations and washings, cells were fixed in PBS with 1% formaldehyde.

The samples were analyzed in a flow cytometer (FACSCalibur, Becton Dickinson). Lymphocytes and AMs were identified and gated by forward and side light scattering properties. In the AM population, quantitative levels of each antigen, expressed as mean fluorescence intensity (MFI), were determined after subtraction of the background fluorescence intensity levels. In the lymphocyte population, the percentages of positively labeled cells in the CD4+ and CD8+ subsets were determined. Isotype matched negative control antibodies always stained less than 1% of CD4+ and CD8+ lymphocytes.

Statistical analysis

The data are presented as medians with upper and lower quartile values (P25–P75) unless otherwise stated. For intraindividual comparisons of data retrieved before and after allergen challenge, Wilcoxon's signed rank test was used. For comparisons of lymphocyte subsets in blood and BAL samples as well as comparisons between patients and controls the Mann–Whitney non-parametric test was used.

Table 1 Description of monoclonal antibodies used for immunophenotyping of macrophages (M) and lymphocytes (L).

Monoclonal antibody specificity	Major function/marker of	Clone	Used for
CD1a	Dendritic cells	NA1/34	M
CD14	LPS-receptor, activation	TüK4	M
CD16	Fc-receptor, activation	DJ130c	M
CD54	Adhesion molecule	6.5B5	M
CD58	Adhesion molecule	BRIC-5	M
CD83	Mature antigen presenting cells	HB15A	M
CD86	Co-stimulation	BU63	M
HLA class I	Activation	W6/32	M
HLA class II	Late activation	CR3/43	M/L
CD69	Early activation	L78	L
CD3	T cells	UCHT1	L
CD4	T cell subset	MT310	L
CD8	T cell subset	DK25	L

Results

Clinical parameters and BAL fluid cell counts

All patients showed early asthmatic reactions (EAR) with peak drops of FEV_{1.0} ranging from 21% to 41% following allergen challenge. Nine subjects developed LAR. The patients showed a significantly increased post-challenge airway reactivity to methacholine, with median PD₂₀ (the dose causing a 20% fall in FEV_{1.0}) values of 450 µg (150–1210) one day before and 135 µg (71–330) two days after the allergen challenge ($P < 0.01$). Cell counts for BAL fluid retrieved from the patients before and after allergen challenge as well as from HC are presented in Table 2. BAL fluid recovery did not differ between the groups. The patients with asthma showed significantly elevated baseline numbers of lymphocytes, eosinophils and mast cells as compared to controls. Following allergen challenge significantly increased numbers of total cells, macrophages and eosinophils as well as increased numbers of mast cells were seen.

AM antigen expression

The AM expression of a number of molecules involved in antigen presentation and interaction with T cells were investigated in BAL fluid retrieved before and after allergen inhalation challenge of 11 individuals with allergic asthma. A representative flow cytometry profile of CD14 expression is shown in Fig. 1. The results, presented as MFI values for each marker, are summarized in Table 3. Due to a very restricted amount of cells available from HC, only markers showing an altered expression following allergen exposure were analyzed in the control material.

The inhalation challenge induced a significantly increased AM expression of the LPS receptor CD14 and the co-stimulatory molecule CD86 (Table 3), whereas the baseline expression of the markers did not differ significantly between asthmatics and HC (data not shown).

Furthermore, tendencies of an increased expression of the antigen presentation-related marker CD1a ($P = 0.09$) as well as a decreased expression of HLA class II ($P = 0.06$) were seen within the AM population following allergen exposure.

Lymphocyte antigen expression

Immunophenotyping of CD4+ and CD8+ lymphocyte subsets in peripheral blood and BAL fluid was performed on the HC as well as before and after

Table 2 Cell counts of BAL fluid retrieved from patients with allergic asthma ($n = 11$) before and after allergen challenge and from healthy controls ($n = 10$).

	Asthmatics before allergen	Asthmatics after allergen	Healthy controls
Total cells ($\times 10^6$)	19.1 (14.7–23.2)	29.8 (19.5–40.4)**	15.9 (12.3–23.2)
Volume of recovered BAL fluid (ml)	179 (160–190)	164 (145–180)	188 (168–204)
Concentration ($\times 10^6/l$) of			
Total cells	106.8 (92.2–129.6)	175.3 (118.9–246.3)**	84.1 (70.3–122.6)
Macrophages	107.1 (82.7–144.5)	167.3 (100.5–292.9)**	78.0 (64.9–114.5)
Lymphocytes	10.7 (5.4–12.9)*	9.8 (5.9–15.5)	5.3 (2.5–7.1)
Neutrophils	1.8 (1.0–2.2)	2.5 (1.1–2.8)	0.7 (0.5–1.3)
Eosinophils	0.2 (0–0.4)*	3.0 (1.9–4.7)**	0 (0–0.2)
Mast cells (cells in 10 visual fields, magnification $\times 160$)	9.0 (4.0–10.0)*	12.0 (6.0–35.0)**	1.5 (0–5.0)

* Indicates $P < 0.05$ compared to healthy controls.

** Indicates $P < 0.01$ compared to before allergen challenge.

allergen challenge on the asthmatic patients (Table 4). The lymphocyte subsets were investigated with regard to expression of the activation markers CD69 (very early activation) and HLA class II (late activation) and the proportions of CD4+ and CD8+ cells out of CD3+ T cells were analyzed.

A tendency towards an increased proportion of CD4+ lung T cells as well as a decreased proportion of CD8+ T cells was seen following allergen challenge. The BAL fluid CD4/CD8 ratio showed a non-significant increase from 2.0 to 2.9. No significant differences were found between patients and controls.

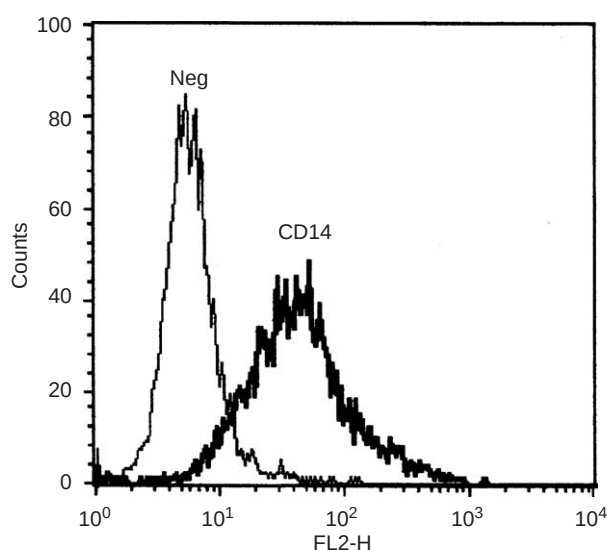


Figure 1 Flow cytometry profiles of one representative patient before allergen challenge demonstrating CD14 staining (bold) compared to negative control staining (thin) in the AM population. The x-axis shows the relative fluorescence on a logarithmic scale and the y-axis shows the number of events.

Asthmatic patients before allergen challenge were compared with HC with regard to the expression of activation markers by T cell subsets in peripheral blood and BAL fluid. There was a significantly higher frequency of cells expressing CD69 in the CD4+ subpopulation of peripheral blood T cells in the patients (Table 4).

Following allergen inhalation challenge there was a significant reduction in the frequency of cells expressing CD69 in CD4+ BAL fluid T cells (Table 4). After allergen challenge there was also significantly fewer cells expressing HLA class II in the CD4+ as well as in the CD8+ subsets of BAL fluid T cells (Table 4). Comparing CD4+ and CD8+ subsets in BAL fluid with the corresponding subsets in peripheral blood, in both T cell subsets there were substantially more cells expressing CD69 and HLA class II in the lungs of the patients (before and after allergen challenge) as well as in the lungs of HC, compared to blood (Table 4).

Discussion

The objective of the present study was to investigate the state of alveolar macrophage (AM) and T cell activation by phenotypic analysis following allergen inhalation exposure of individuals with mild atopic asthma. We found that induction of airway inflammation was associated with a reduced frequency of pulmonary T cells expressing the activation marker CD69 and the late activation marker HLA class II as well as an increased quantitative expression of CD14 and CD86 within the AM population, compared to before challenge. These alterations of the phenotype pattern are consistent with a recruitment of immature cells to the airways, but could also, for AMs, be an effect of

Table 3 Expression of surface markers for activation, adhesion and co-stimulation by alveolar macrophages retrieved from asthmatic patients before and after allergen inhalation challenge.

	Before allergen challenge	After allergen challenge	P
CD1a	1.0 (0.3–1.5)	1.6 (1.0–2.3)	0.09
CD14	50.7 (29.0–66.3)	110.6 (73.9–115.4)	<0.01
CD16	203.9 (135.3–237.3)	190.7 (109.3–136.4)	0.42
CD54	61.9 (48.7–72.8)	60.6 (53.5–78.8)	0.78
CD58	5.0 (3.5–7.6)	4.1 (2.5–6.4)	0.59
CD83	0.8 (0.2–1.7)	0.8 (0.3–2.1)	0.44
CD86	73.4 (58.9–83.4)	93.4 (60.9–115.1)	<0.05
HLA class I	1360 (1057–1710)	1101 (816–1336)	0.1
HLA class II	1425 (1179–1723)	1030 (744–1295)	0.06

The mean fluorescence intensity (MFI) values for each marker was determined by FACS analysis. The table depicts median (P25–P75) for the respective MFI values in the group of 11 patients.

Table 4 Immunophenotyping of PBL and pulmonary (BAL) lymphocyte subsets retrieved from asthmatics before and after allergen inhalation challenge and from healthy controls.

% Lymphocytes		PBL		BAL			
		No allergen (n = 11)	Allergen (n = 11)	Healthy (n = 10)	No allergen (n = 9)	Allergen (n = 7)	Healthy (n = 10)
CD69	CD4	1.5 (0.7–2.2)*	1.3 (0.9–3.1)	0.6 (0.5–0.8)	71.0 (64.8–76.1)	61.0 (49.2–64.4)**	63.8 (60.0–78.0)
	CD8	3.3 (3.0–6.3)	2.6 (2.0–4.7)	2.0 (1.4–3.2)	82.4 (78.5–84.7)	83.5 (73.6–85.9)	88.0 (80.7–90.0)
HLA class II	CD4	3.6 (3.0–6.3)	5.0 (2.7–6.2)	3.6 (2.2–3.9)	50.0 (18.4–59.5)	17.8 (12.2–22.1)**	42.0 (25.0–56.8)
	CD8	11.2 (4.5–13.8)	7.7 (4.7–16.4)	8.9 (4.0–17.2)	48.9 (23.7–53.5)	16.4 (7.9–25.5)**	39.6 (28.4–45.7)

The data illustrate the median (P25–P75) percentage of lymphocytes in CD4+ and CD8+ subsets positive for CD69 and HLA class II, respectively.

*Indicates $P < 0.05$ compared to healthy controls.

**Indicates $P < 0.05$ compared to before allergen challenge.

altered expression induced by allergen exposure and could be of importance in the regulation of the allergic airway inflammation. Although we cannot exclude the possibility that the results may have been influenced by the methacholine challenge one day before the allergen challenge, we deem this unlikely since it has been shown that methacholine inhalation does not evoke an inflammatory cell infiltrate in BAL fluid.¹⁸

In health the AM is considered to act primarily immunosuppressive, while certain pulmonary inflammatory conditions such as allergic asthma is associated with a shift of the AM function to a T cell stimulatory function.^{5,12,13} An important factor in T cell activation is the co-stimulatory signal(s) delivered by the APC during antigen presentation, for instance by the CD80/CD86 (B7-1/B7-2) molecules interacting with CD28 on the T cell surface. Inhibition of this specific co-stimulatory pathway has been shown to prevent allergen-induced activation of airway-derived T cells in atopic asthmatic individuals.^{19,20} A previous study demonstrated an induced monocyte expression of CD86 and CD1a following allergen- or Th2 cytokine stimulation in vitro, as well as overexpression of CD86 and CD1a (traditionally seen as a marker of dendritic cells) by AMs from asthmatics.⁵ We here show an upregulated AM expression of CD86 one day after allergen exposure in vivo, a phenotypic shift indicating an increased accessory cell function within the AM population. The phenotypic analysis furthermore showed a post-challenge tendency of an increased AM expression of the antigen presentation-associated marker CD1a.

The increased CD14 expression may be explained by alterations in AM subsets, following an influx of blood monocytes.^{21,22} This is consistent with our previous demonstration of an altered phenotype, including an increased expression of CD11b and CD14, of AM in patients with asthma after repeated low-dose allergen inhalation challenge.²³ Other investigators have also found that the frequency of monocytes is increased in asthmatic airways.^{24,25} However, it is also possible that the CD14 expression on cells already resident in the lungs has been upregulated, since it was shown that allergen exposure leads to increased CD14 expression on monocytes.²⁶ CD14 has been implicated as a candidate gene for allergic diseases, since it is localized in the chromosome 5q31-33 region, which has been linked to the control of IgE levels, asthma and bronchial hyperresponsiveness.²⁷ Several polymorphisms in the CD14 promoter have been associated with atopy in various populations.²⁸ An altered CD14 expression may well lead to a change in endotoxin response, since CD14 together with

TLR4 is a receptor for lipopolysaccharide (LPS). In patients with allergic asthma there is increased expression of CD14 in the airways following allergen challenge.⁸ In our low-dose allergen challenge study AM CD14 expression correlated negatively with lung function measured as post-challenge FEV₁.²³ A polymorphism in the promoter region of CD14 has also been shown to be linked to soluble CD14 levels and total IgE levels.²⁹ One mechanism behind this could be an altered reactivity to bacterial products, affecting Th cell differentiation. Furthermore, CD14 has been suggested to have a role in regulating apoptosis in monocytes, with increased expression promoting survival.³⁰ These findings suggest several ways in which CD14 expression could be associated with pathogenic events in allergic asthma.

Comparing the baseline lymphocyte expression of activation markers in patients and HC, we found a higher percentage of cells expressing CD69 in CD4+ PBL lymphocytes retrieved from patients before allergen challenge. A subset of blood CD4+ T cells thus appears to be activated even in the absence of symptoms of asthma. A sub-clinical activation of the immune system is further supported by our findings of elevated baseline levels of BAL fluid lymphocytes and eosinophils in the patients (Table 2). Our previous observation of a significantly altered TCR V gene expression of BAL fluid T cells derived from individuals with asthma before allergen challenge is also in line with these results.³¹ The finding that BAL T cells, compared to PBL T cells, more frequently express CD69 and HLA class II in patients as well as in controls, is in congruence with previous reports.³² The significantly decreased proportion of cells expressing early (CD69) as well as late (HLA class II) activation markers in the CD4+ BAL fluid T cells after allergen provocation suggests that the T cells accumulated at this early stage are non-activated.

Our findings indicate that in vivo allergen inhalation challenge in subjects with allergic asthma renders a pulmonary accumulation of immature monocytes and CD4+ T cells together with an altered AM phenotypic pattern indicating the adoption of a T cell stimulatory function, likely to be of importance for the further development of the allergic airway inflammation.

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