

Original article

Dendritic cell number is related to IL-4 expression in the airways of atopic asthmatic subjects

Background: Airway dendritic cells are essential for stimulating naive T cells in response to inhaled antigen and for the development of allergic sensitization. IL-4 *in vitro* can distinguish dendritic cell lines from peripheral blood mononuclear cells. Our study had the following aims:

- 1) to compare the distribution of CD1a+ dendritic cells and IL-4+ cells, in the bronchial mucosa of asthmatics and controls
- 2) to determine the relationship between the numbers of CD1a+ dendritic cells and IL-4+ cells in the bronchial mucosa of asthmatics
- 3) to determine whether CD1a+ cells express the IL-4 receptor.

Methods: Twenty atopic asthmatic and eight normal subjects were studied. In each subject, bronchoscopy with bronchial biopsies was performed. CD1a, IL-4, and IL-4 receptor expressions were evaluated by immunohistochemistry.

Results: The number of CD1a+ and IL-4+ cells was significantly higher in asthmatics than controls. The number of CD1a+ cells was positively correlated to the number of IL-4+ cells. Bronchial biopsy serial section studies showed that CD1a+ cells express the receptor for IL-4.

Conclusions: These results suggest that an increased amount of IL-4 may play a physiopathologic role in maintaining the dendritic cell pool *in vivo*. Therefore, because of possible IL-4 activity on antigen-presenting cells in T-cell immune responses to allergens, an important new role of IL-4 in asthma inflammation can be envisaged.

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Dendritic cells (DC) are a family of professional antigen-presenting cells (APC) responsible for the activation of naive T cells and the generation of primary T-cell responses (1). For these functions, DC residing in nonlymphoid tissues need to be activated to initiate the differentiation process. DC maturation is characterized by profound changes in MHC class II distribution, antigen-processing capacity (2), and expression of costimulatory molecules (3), and by a marked rearrangement of adhesion molecules that is likely to allow DC migration to lymphoid organs (4). DC maturation is probably controlled by microenvironmental signals. However, little is known about the factors and mechanisms regulating DC cell cycle, life span, and functional activity. Cytokines control DC movement (4), survival (5), and APC activity (6, 7), but the fine biochemical mechanisms underlying these effects are unknown (8).

The presence of a chronic inflammatory process in the airways of asthmatic patients (9) has drawn

attention to the mechanisms responsible for the initiation and maintenance of the immunologic response. DC are thought to play a leading role in the local T-cell activation observed in asthmatic patients. In fact, their antigen-presenting capacity is much greater than that of any other potential APC (1, 10), and it has been suggested that in asthma they can selectively activate Th2-like lymphocytes both *in vitro* and *in vivo* (11–13). Priming of naive CD4+ T cells in the presence of IL-4 can develop Th2 effector cells. After secondary stimulation, Th2 cells secrete large amounts of cytokines such as IL-4 (14).

Our study had the following aims:

- 1) to study the distribution of CD1a+ DC and IL-4+ cells in the bronchial mucosa of asthmatic patients
- 2) to determine whether the number of CD1a+ cells correlates with that of IL-4+ cells
- 3) to determine whether CD1a+ cells express the IL-4 receptor.

Material and methods

Subjects

Twenty atopic subjects with mild to moderate stable asthma and eight healthy volunteers were included in the study (Table 1). All subjects were lifetime non-smokers.

The diagnosis and classification of asthma were based on history, symptoms, clinical features, and medication requirement according to international guidelines (15). All the asthmatics had increased airway responsiveness to methacholine (cumulative concentration producing a fall in forced expiratory volume in 1 s [FEV₁] of 20% from baseline [PC₂₀] <16 mg/ml). All the asthmatics were atopic, as defined by positive skin prick tests to common aeroallergens.

Respiratory symptoms of the asthmatic subjects were controlled with β_2 -adrenergic drugs on a continuous

basis or on demand. None of the subjects had taken inhaled or oral corticosteroids for at least 6 months before the study.

Normal controls were not taking any form of medication, had no history of asthma or other allergic diseases, had negative skin prick tests, and showed a methacholine PC₂₀ of >64 mg/ml.

The study conformed to the Declaration of Helsinki and was approved by the University of Parma ethical subcommittee. Written informed consent was obtained from each subject.

The study was conducted outside the pollen season. After selection, all asthmatic subjects were studied for a preliminary 2-week run-in period, during which any asthmatic symptoms were recorded on diary cards. In addition, patients were asked to record their β_2 -agonist use and peak expiratory flow (PEF) as the best of three pretreatment measurements taken twice daily. PC₂₀ methacholine was measured during the run-in period at least 3 days before fiberoptic bronchoscopy, which was done at the end of the run-in period.

Table 1. Clinical and demographic characteristics of study subjects

Subject no.	Age (years)	Sex	Atopy*	Baseline FEV ₁ [°] (% pred)	PC ₂₀ [§] (mg/ml)	Regular medication [×]
Asthmatics						
1	18	M	Yes	99	5.05	B p.r.n.
2	23	M	Yes	116	4.05	B
3	20	M	Yes	97	1.22	B p.r.n.
4	20	M	Yes	110	5.20	B p.r.n.
5	26	M	Yes	108	0.75	B
6	21	M	Yes	116	11.85	B
7	21	M	Yes	113	5.65	B p.r.n.
8	45	F	Yes	121	2.49	B p.r.n.
9	50	M	Yes	86	2.14	B
10	28	F	Yes	101	0.17	B
11	18	M	Yes	93	0.24	B p.r.n.
12	18	M	Yes	93	11.3	B p.r.n.
13	18	M	Yes	102	4.76	B p.r.n.
14	42	F	Yes	112	6.90	B
15	28	M	Yes	119	2.58	B p.r.n.
16	26	M	Yes	87	0.62	B
17	21	M	Yes	85	0.15	B
18	20	M	Yes	115	13.5	B p.r.n.
19	24	M	Yes	82	1.12	B
20	19	M	Yes	95	0.16	B
Controls						
1	22	M	No	114.9	>64	—
2	25	F	No	116.7	>64	—
3	26	F	No	101	>64	—
4	23	M	No	102.6	>64	—
5	27	M	No	115.5	>64	—
6	29	M	No	92.6	>64	—
7	22	F	No	112.5	>64	—
8	23	F	No	109	>64	—

* As determined by positive skin prick tests (wheal diameter >3 mm) to at least two common inhalant allergens.

° Asthma severity score.

§ Forced expiratory volume in 1 s.

§ Cumulative concentration of methacholine causing 20% drop in FEV₁ from postsaline values.

× β_2 : β_2 -agonists; p.r.n. as needed (pro re nata).

Fiberoptic bronchoscopy

Fiberoptic bronchoscopy was performed according to our previously published protocols (16–18) and conformed to current US National Heart, Lung, and Blood Institute guidelines (19).

Subjects received 0.5 mg atropine sulfate and 10 mg diazepam intramuscularly 30 min before the procedure. Local anesthesia was obtained by 2% lignocaine 15 min before bronchoscopy. An additional aliquot of 2% lignocaine was aerosolized into the upper airways and applied topically to the pyriform sinuses and vocal cords to prevent coughing and as a local anesthesia. No other drugs were administered before or during bronchoscopy.

Fiberoptic bronchoscopy was introduced through the nose or transorally (Olympus BF, Type 1T 10; Olympus Optical Co., Tokyo, Japan). Three to five mucosal biopsy specimens were taken with alligator forceps at the subcarinae in the middle and lower lobes of the right lung.

Processing of bronchial biopsies and staining procedure

Mucosal biopsies were immediately transferred into ice-cooled acetone, containing the protease inhibitors iodoacetamide (20 mM) and phenylmethylsulfonyl-fluoride (PMSF) (2 mM) for fixation; stored at –20°C for 24 h; and then processed into the water-soluble resin, glycolmethacrylate (GMA) (Polysciences, Northampton, UK), for embedding (20).

Sections of 2 μ m were cut by ultramicrotomy, floated on 0.2% (v/v) ammonia solution for 1 min, picked up onto poly-L-lysine-coated slides, and dried at room temperature for 1–4 h. Immunostaining used mouse monoclonal antibodies against CD1a, an antigen widely

used as a marker for tissue DC (Dako, Glostrup, Denmark) and IL-4 (B-H18, Biosource International, Camarillo, CA, USA). We also used rabbit polyclonal antibody against the peptide corresponding to amino acids 801–820, mapping at the carboxy terminus of IL-4 receptor- α of human origin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Immunostaining applied to GMA-embedded tissue has been previously described by Britten et al. (20). Briefly, slides were pretreated with a solution of 0.1% (w/v) sodium azide and 0.3% (v/v) hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity. After three 5-min washes in Tris-buffered saline (TBS), pH 7.6, blocking medium consisting of Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 1% bovine serum albumin was applied for 30 min. Sections were then incubated with the primary antibody for 16–20 h overnight at room temperature. After overnight incubation, the sections were washed in TBS. Anti-CD1a- and IL-4-bound antibodies were then labeled with biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA, USA). Anti-IL-4 receptor- α -bound antibodies were labeled with biotinylated antirabbit IgG (Vector Laboratories, Burlingame, CA, USA). Labeling was performed during a 2-h incubation and made visible by a streptavidin-biotin-horseradish-peroxidase complex detection system (Dako, Glostrup, Denmark). Aminoethylcarbazole was applied as chromogen, resulting in a red reaction product. The sections were counterstained with Mayer's hematoxylin. Appropriate unrelated antibodies of the same isotypes and concentrations as the primary antibodies were applied to negative control sections.

Assessment and quantification of immunohistochemical staining

Light microscopy was done with a Leica microscope (Leica DMLB, Wetzlar) at a magnification of $\times 400$. CD1a- and IL-4-positive cells in the lamina propria were counted in a zone 100 μm deep in the bronchial mucosa along the length of the epithelial basement membrane, and expressed as number of positive cells per mm^2 of lamina propria. The length of epithelial basement membrane and area of lamina propria were calculated by an image processing and analysis system (Leica Q500C, Leica Cambridge Ltd, Cambridge, UK).

In the lamina propria, sequential sections were compared by an image processing and analysis system (Leica Q500C, Leica Cambridge Ltd, Cambridge, UK). In eight biopsies from the asthmatic group and six biopsies from the control group, CD1a immunoreactivity was paired with adjacent sections separately stained for the IL-4 receptor. At least three fields at $\times 400$ magnification were compared for each pair of adjacent sections to enable the IL-4 receptor to be localized to the CD1a cell type. Biopsies used for the study of sequential sections were chosen only if CD1a

immunoreactivity was present. These subjects did not differ from their respective group members in age, sex, FEV₁ % predicted, or PC₂₀.

Data analysis

Values are presented as mean $\pm$ standard deviation (SD). Differences between unpaired data were calculated by Student's *t*-test. The correlation between number of CD1a-positive cells and number of IL-4-positive cells was assessed by Spearman's rank correlation coefficient. Statistical significance was assumed when $P \leq 0.05$.

Results

Demographic and functional characteristics of subjects

The demographic and functional characteristics of the study population are reported in Table 1. The mean ages of asthmatic patients (25.30 ± 9.43 years) and normal subjects (24.63 ± 2.56 years, $P = 0.8451$) were similar. The pulmonary function of patients with asthma as assessed by FEV₁ ranged from 82% to 121% of predicted values. Asthmatic patients were classified as mild to moderate. The pulmonary function of normal subjects was within the normal range. Asthmatics had higher responsiveness to methacholine (PC₂₀ 3.99 ± 4.12 mg/ml) than controls (PC₂₀ < 64 mg/ml). Bronchoscopy and bronchial biopsy were performed successfully and were well tolerated by all subjects.

Biopsy findings

Immunoreactivity for the CD1a marker, IL-4, and the IL-4 receptor was present in the bronchial mucosa of both asthmatic and control subjects. As our previous study demonstrated (21), T cells, mast cells, and eosinophils all expressed IL-4. No immunostaining was identified on negative control sections.

In the lamina propria, the number of CD1a+ cells was significantly increased in the asthmatic group when compared to control subjects (11.73 ± 3.90 vs 1.44 ± 1.07 , $P < 0.001$) (Fig. 1). The number of IL-4+ cells was also significantly increased in the asthmatic group when compared to control subjects (158.20 ± 45.97 vs 23.50 ± 11.99 , $P < 0.001$) (Fig. 2). A significant positive correlation was found between the number of CD1a+ cells and the number of IL-4+ cells in the asthmatic group ($r = 0.658$, $P < 0.002$) (Fig. 3).

IL-4 receptor immunoreactivity was localized on different cell types, such as epithelial cells, endothelial cells, fibroblasts, muscle cells, mast cells, macrophages, and T cells (data not shown). Examination of serial sections was performed in a subgroup of eight asthmatics and six control subjects. In the asthmatics, comparison of adjacent sections showed that in the

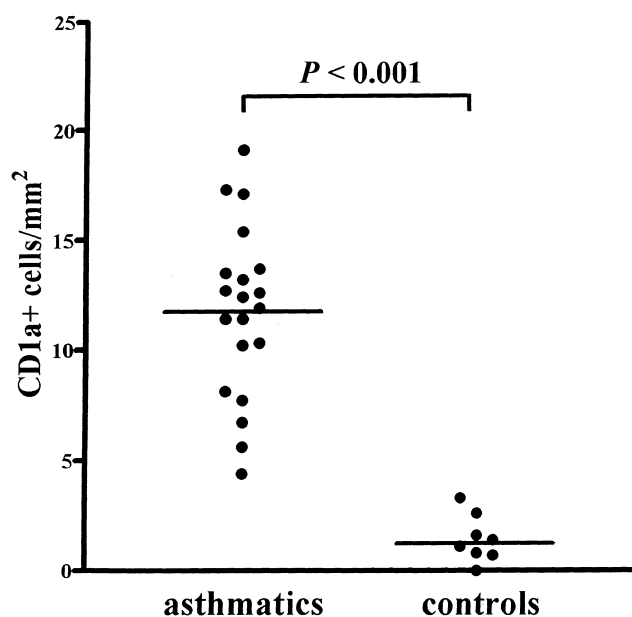


Figure 1. CD1a+ cells in lamina propria of asthmatic and control subjects. Results are expressed as number of positive cells per mm² lamina propria. Horizontal bars represent mean values.

lamina propria a median of 47.5% (18–61%) of CD1a+ cells were positive for antibody to IL-4 receptor. In the biopsies from control subjects, we were able to colocalize the IL-4 receptor immunoreactivity to a few CD1a+ cells in only one case. A representative example of bronchial biopsy adjacent sections stained with both anti-CD1a and anti-IL-4 receptor is shown in Figs. 4a and b.

Discussion

The results of this study indicate that:

- 1) the bronchial mucosa of asthmatic patients is characterized by an increased number of both CD1a+ and IL-4+ cells compared to control subjects
- 2) the number of CD1a+ cells is positively correlated with the number of IL-4+ cells
- 3) CD1a+ cells express the receptor for IL-4.

On the basis of these findings, we suggest that IL-4 upregulation may contribute to DC, recruitment at the local microenvironmental level in asthmatic airways.

A highly specific monoclonal antibody against the CD1a antigen was used to identify DC, since other cells known to express this marker are either present in the airways at very low frequency (B cells) or absent altogether (cortical thymocytes). In agreement with other studies (22, 23), we used the CD1a antigen as a marker for airway DC. There are several ways to identify DC, including morphologic and functional methods. None of them allow the identification of all

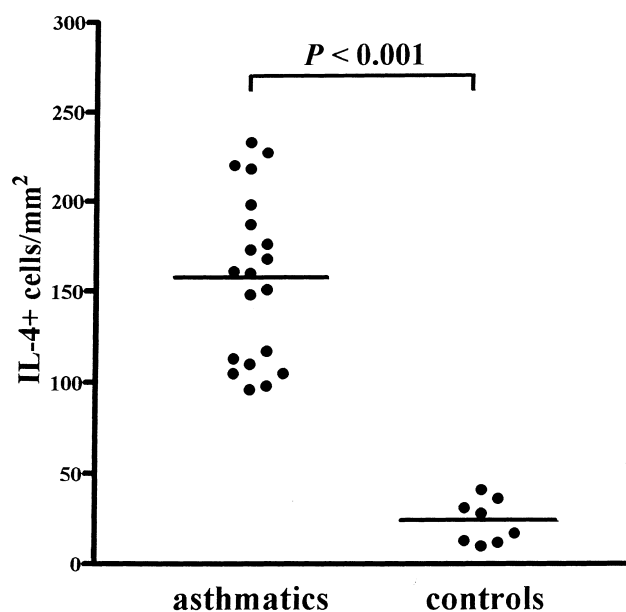


Figure 2. IL-4+ cells in lamina propria of asthmatic and control subjects. Results are expressed as number of positive cells per mm² of lamina propria. Horizontal bars represent mean values.

types of DC. The detection of CD1a+ cells by immunohistochemical staining is a reliable and simple method to identify DC in tissues. However, this method identifies only a subgroup of DC, and not all DC; therefore, we have used the term “CD1a+ DC” instead of “DC” throughout the text. Moreover, it has been shown *in vitro* that human monocytes cultured in the presence of GM-CSF can upregulate CD1a antigen (24). This event is not verified in *in vivo* studies at the

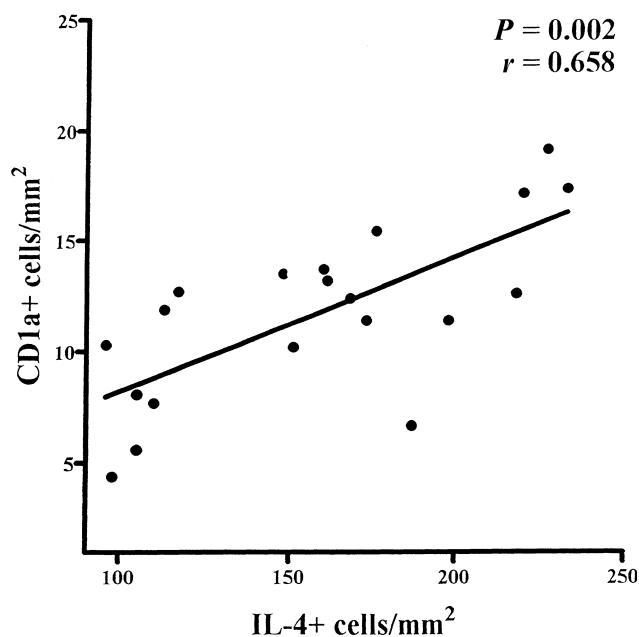
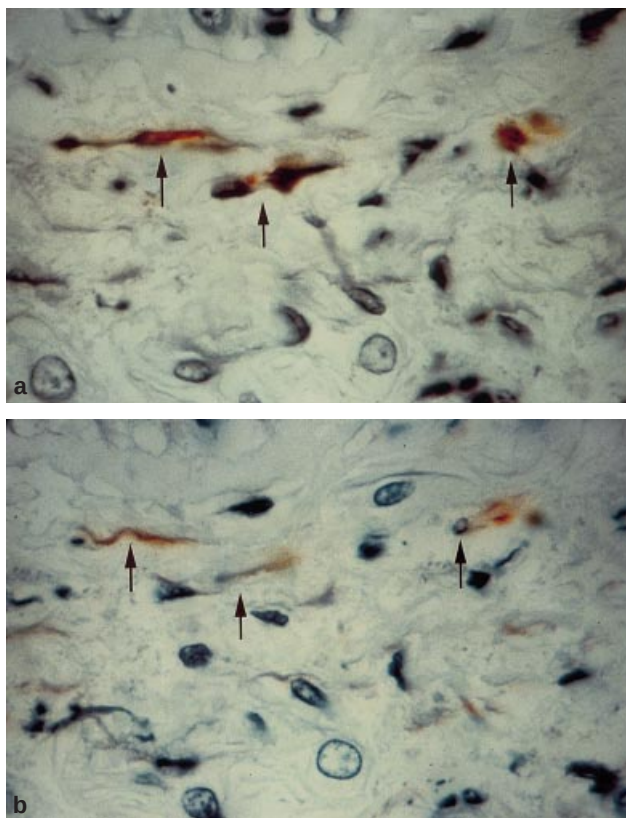


Figure 3. Linear correlation between CD1a+ and IL-4+ cells in lamina propria of asthmatic subjects.



Figures 4a and b. Sequential 2- μ m sections of bronchial mucosa from asthmatic subjects stained with mAb NA1/34 to CD1a (Fig. a), and polyclonal antibody to human IL-4R α (Fig. b). Arrows show cells stained with both antibodies, indicating colocalization of IL-4R α to CD1a cells.

moment. The possibility that CD1a⁺ cell populations include activated monocytes may lead to an overestimation of DC numbers.

We found that a higher number of DC are present in the airways of asthmatic patients than in controls. This finding is consistent with other recent reports indicating that DC are more numerous in the airways of atopic asthmatics than atopic nonasthmatic subjects (11), in the airway of atopic and nonatopic asthmatics than healthy control subjects (22, 25, 26), and in the nasal mucosa of grass-pollen-sensitive patients with allergic rhinitis (27).

DC are potent APC for inducing the activation of T cells, including either resting or activated, naive or memory T cells. Recently, Tunon-de-Lara et al. have shown that the proportion of DC expressing Fc ϵ RI α was significantly increased in asthmatics (22). In addition, there is evidence that DC are recruited into the airway epithelium during inflammatory responses to a broad spectrum of stimuli (28), and are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen (29). The activation of Th2 cells in allergic diseases results in an increased amount of IL-4 expression.

Increased IL-4 expression could be responsible for an increase in the number of local DC. The hypothesis that IL-4 expands the local DC pool is suggested by our findings that DC express the receptor for IL-4, and also that an increase in the number of DC is paralleled by an increased IL-4⁺ cell number. This hypothesis is supported by studies in man and rodents showing that DC and monocytes derive from a common precursor. Strong evidence suggests that DC may derive from the myelomonocytic lineage soon after the monocyte differentiation state (30). Moreover, evidence shows that even mature macrophages may convert into potent DC if signals causing an increase in intracytoplasmic cyclic AMP are provided. The local microenvironment could be decisive as to which phenotype is expressed (31). Under serum-free conditions, monocytes can lose their specific CD14 marker, differentiating into a DC-like phenotype under the influence of IL-4. Using granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-4, Sallusto & Lanzavecchia have established DC lines from blood mononuclear cells that maintain the antigen-capturing and -processing capacity characteristic of immature DC *in vivo* (32). A combination of GM-CSF and IL-4 was shown to provide the best conditions for the generation of cells with the phenotypic characteristics and functional properties of DC (high expression of CD1, class II, and B7, and high stimulatory capacity in allogeneic and autologous mixed leukocyte reaction). In allergic airway inflammation, IL-4 is an important cytokine and could be involved not only in the induction of IgE synthesis and other well-known biologic effects, but also in the upregulation of the DC pool.

In conclusion, our data show that the bronchial mucosa of asthmatic patients is characterized by an increased number of both CD1a⁺ and IL-4⁺ cells as compared to control subjects, and that the number of CD1a⁺ cells is positively correlated with that of IL-4⁺ cells. Finally, CD1a⁺ cells express the receptor for IL-4. These results suggest that an increased amount of IL-4 plays a physiopathologic role in maintaining the DC pool *in vivo*. Because of its activity on APC in T-cell immune responses to allergens, an important new role of IL-4 in asthma inflammation should be considered.

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