

Respiratory Tract Dendritic Cells in Paediatric Asthma

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Running title: Airway dendritic cells in paediatric asthma

Abstract

Background

Airway dendritic cells (DC) are critical mediators of lung inflammation in asthma, but the characteristics of DC in the airways of healthy children, and children with asthma, are currently unknown.

Objective

We sought to identify changes in DC subset distribution and activation profile in paediatric asthma by using flow-cytometry to analyze induced sputum samples obtained from healthy and asthmatic children.

Methods

Lung function and atopic status were determined by spirometry and skin prick testing. Induced sputum samples were analyzed using 7-color flow-cytometry to identify airway DC populations (lineage⁻ HLA-DR⁺ sputum cells expressing either CD11c as conventional DC or CD123 as plasmacytoid DC).

Results

Sputum samples containing lower airway plugs were obtained from 10 healthy children and 8 children with asthma. Lineage⁻ HLA-DR⁺ DC were successfully identified in all samples, and DC comprised a significantly higher proportion of sputum cells in children with asthma compared with age-matched healthy controls (1.29% vs. 0.67%, $p=0.02$). DC expression of the co-stimulatory marker CD86 was significantly reduced in asthmatic children (73.4% vs. 59.7%, $p=0.04$). Sputum DC also included numerous CD1c⁺ cells (mean 57% of the total DC population) and low frequencies of cells expressing the subset markers CD141 or CD123, although the proportions of these did not differ between groups.

Conclusions

Airway DC can be identified and characterized non-invasively by using flow-cytometry to analyze paediatric sputum samples. Our data reveal that children with steroid-treated asthma exhibit increased frequency of airway DC with reduced expression of the co-stimulatory marker CD86, suggesting altered trafficking and/or maturation of these cells either due to asthma or steroid therapies.

Introduction

Airway dendritic cells (DC) are critical mediators of immune responses in the lung by virtue of their ability to sample, process and present inhaled antigens to T-cells [1-3]. DC are therefore thought to play a key role in driving airway inflammation in asthma, since depletion of DC in rodent models prevents the induction of Th2 responses to an airway sensitization regime [4], and instillation of allergen-loaded DC into the airways of healthy mice induces an asthma-like response [5]. Accordingly, modulation of airway DC responses to inhaled antigens has been proposed as a promising future approach to asthma therapy [6].

Populations of pulmonary DC have previously been identified by flow-cytometric analysis of lung tissue obtained from adults [7, 8]. Two major subsets of DC are thought to reside in the healthy human lung; conventional DC (cDC), which express HLA-DR and CD11c, and plasmacytoid DC (pDC), which express HLA-DR and CD123. Blood dendritic cell antigens (BDCAs) have also been used by some investigators to further classify lung DC into subgroups; CD1c/BDCA1⁺ DC, and CD141/BDCA3⁺ DC [7]. The precise functional roles of these subsets in the human lung are unclear; on the basis of Toll-like receptor (TLR) expression it was provisionally suggested that cDC are predominantly involved in bacterial responses, with variable TLR expression within the CD1c⁺ and CD141⁺ subgroups, and that pDC are primarily involved in viral responses [7], these observations are organ specific as TLR expression on splenic DC subsets differs from that on DC in the lung [9].

More recent studies have identified subsets of airway DC in broncho-alveolar lavage fluid (BALF) [10] and in samples of induced sputum (IS) from adults with asthma, obtained both pre- and post-challenge with allergen [11, 12]. In these flow cytometry-based studies, the majority of airway DC were found to exhibit a myeloid phenotype (60-80% CD11c⁺), and the total DC population was observed to increase in the airway of atopic asthmatic patients after allergen inhalation, consistent with findings from immunohistochemical studies of bronchial [13] and nasal [14] mucosa. It is therefore clear that multiple DC types can populate the human lung in both health and disease, but the relative contributions of these subsets to the induction of airway inflammation in children is currently unknown.

Previous studies have reported that cDC frequency and activation profile is significantly altered in adults with asthma and can be further modulated by allergen inhalation. Using segmental allergen challenge, Bratke *et al* reported that cDC in BAL expressed higher levels of co-stimulatory markers CD40, CD80, CD83 and CD86 than did their counterparts in blood, and these cells displayed a significant decrease in CD83 expression after allergen

challenge [10]. Lavage samples differ from IS, as sputum induction samples central airways [15], and contains a reduced proportion of macrophages compared to BAL [16]. McCarthy *et al* reported that human respiratory-tract DC expressed increased levels of CD86 in atopic asthma that were further up-regulated by allergen inhalation [11], and Dua *et al* showed that CCR6 expression (a potential marker of recruitment) was increased on cDC in induced sputum following allergen challenge [12]. These data indicate that airway DC phenotype and frequency are substantially altered in adults with asthma, but to date, asthma-related changes in airway DC populations, subsets and co-stimulation markers have not been determined in children. This is largely due to the technical difficulties associated with accessing the lower airways in children, as broncho-alveolar lavage requires general anaesthesia, and the low numbers of cells that can be non-invasively obtained using IS for *ex vivo* analysis. However, multicolor flow-cytometry makes it possible to analyze low-volume clinical samples containing a mixture of cell types for small populations of cells such as airway DC. We therefore sought to use 7-color flow-cytometry to test our hypothesis that DC frequency and maturation are increased in the sputum of children with asthma.

Methods

Participants

Children with physician-diagnosed asthma were recruited from the respiratory outpatient clinic at The Royal London Children's Hospital. Diagnosis of asthma was confirmed from electronic case records. Healthy children and children with asthma were also recruited from schools in east London. Written informed consent was obtained from the parents of all participants (Research ethics committee references 11/LO/1732 and 13/LO/0440).

Skin prick testing

Children with asthma had all undergone skin prick testing as part of routine clinical care and the results of these tests were retrieved from electronic records. Healthy schoolchildren underwent skin prick testing to common aeroallergens (house dust mite, tree pollen, grass pollen, cat dander, dog dander, aspergillus) plus histamine and saline controls (all reagents from Allergopharma Joachim Ganzer KG, Reinbek, Germany). Reagents were applied to the skin using the Multitest-II (Lincoln Diagnostics, Decatur, IL, USA) and a positive test was determined as a skin wheal 2mm greater than histamine control at 15min.

Spirometry and induced sputum

Spirometry was performed according to ERS/ATS criteria [17] using a Microlab spirometer (Care Fusion, Kent, UK). Lung function was measured at baseline and at 15min after inhalation of 400µg salbutamol administered via metered dose inhaler and large volume spacer. Standard deviation scores (z-scores) for forced expiratory volume in 1 sec (FEV₁) and forced vital capacity (FVC) were calculated using Global Lung Initiative software [18]. Children with asthma underwent sputum induction during a clinician-assessed period of disease stability (no exacerbation requiring oral corticosteroids during the preceding month). Sputum induction and processing were performed as previously described [19, 20] using nebulized 4.5% saline administered via a Multisonic Profi nebuliser (Schill Medical, Probstzella, Germany) for a maximum of 20min, as per ERS guidelines for sputum induction in children [21]. FEV₁ and FVC were measured at 5min intervals to monitor for risk of bronchospasm. Samples were placed on ice and processed within 1h of induction.

Sputum processing and leucocyte differential

Sputum was processed by selecting for cell-rich plugs of airway mucus as per the method of Pizzichini *et al* (22), where airway plugs are considered as quasi-cylindrical muco-cellular casts of peripheral airways. Samples lacking visible airway plugs were discarded. Whole-sputum samples were processed as previously reported [19]. Briefly, whole-sputum samples were diluted in phosphate buffered saline (PBS) and agitated. Airway plugs were selected on visual inspection. 0.1% DTT (Dithiothreitol, Sigma-Aldrich, St Louis, MO, USA) was added and the samples were vortexed, then gently agitated on ice for 15 min to facilitate mucolysis. Samples were then passed through a gauze filter and centrifuged at 10000 rpm for 10 min. Following this, pellets were re-suspended in PBS. An aliquot was transferred into a cytospin funnel and spun at 1500 rpm for 3 min. Leucocyte differential counts were calculated by observing 400 leucocytes at x40 magnification and determining the percentage of different cell types present.

Antibody labelling and flow cytometry

Sputum cells were suspended in FACS buffer (PBS containing 2% fetal calf serum, 0.02% sodium azide, 1mM EDTA) and then labelled for 20min on ice with monoclonal antibodies against human CD1c (clone L161, PerCP-Cy5.5), HLA-DR (L243, PE-Cy7), CD86 (IT2.2, APC), CD11c (3.9, AmCyan), CD123 (9F5, PE), CD141 (AD5-14H12, VioBlue), and a dendritic cell exclusion cocktail of antibodies against CD3, CD14, CD16, CD19, CD20 and CD56 (clones UCHT1, HCD14, HIB19, 2H7 and HCD56 respectively, FITC), which permits the exclusion of T-cells, macrophages, neutrophils, B-cells and natural killer cells respectively. All antibodies and corresponding isotype controls were obtained from BioLegend (San Diego, CA), except for anti-CD123 from BD Biosciences (Cardiff, UK) and anti-CD141 from Miltenyi Biotec (Bisley, UK). After labelling, the cells were washed in

FACS buffer, centrifuged for 5min at 1700rpm, the supernatants discarded, and the cell pellets fixed in 1% paraformaldehyde. Data were acquired within 2h of antibody labelling using a BD LSR II flow cytometer with FACSDiva v6.1.03 software (Becton Dickinson, Oxford, UK). Data were analyzed by an investigator blinded to asthma status (N.M.) using WinList 6.0 (Verity Software House, Maine). Dendritic cells (DC) were identified as HLA-DR⁺ sputum cells that lacked expression of markers for alternative lineages (Lin^{-dim} cells; CD3- CD14- CD16- CD19- CD20- CD56-, figure 1). Conventional DC were defined *a priori* as Lin⁻ HLA-DR⁺ CD11c⁺ cells, and plasmacytoid DC were enumerated as Lin⁻ HLA-DR⁺ CD11c⁻ CD123⁺ cells. CD86 was selected as an activation marker on the basis of a previous report [11]. All DC surface marker expression was determined relative to isotype-matched control antibodies, except for CD11c expression, which was determined relative to the fluorescence levels of non-myeloid sputum cells within the same sample.

Statistical analysis

Data are presented as mean ± standard error. A D'Agostino and Pearson omnibus normality test was performed to test the distribution of data. For parametric data, unpaired Student's t-tests and Fisher's exact test were used to determine differences between groups. For non-parametrically distributed data, the Mann-Whitney test was used to determine difference between groups. Potential associations were tested by calculating Pearson's correlation coefficient (parametric) and Spearman's correlation (non-parametric). Analyses were performed using Prism 5.00 for Windows (GraphPad Software, CA, USA). P<0.05 was considered significant.

Results

Study participants

Sputum samples containing visible airway plugs were obtained from 10/21 healthy children and 8/12 of asthmatic children who enrolled in the study. The healthy control and asthmatic groups did not differ significantly in age, atopic status or lung function score (table 1). Within the asthma group, 2 children were at BTS step 1 treatment (inhaled salbutamol only), 5 were at BTS step 3 (inhaled corticosteroid plus long-acting inhaled beta agonist) and one child was at BTS step 4 (inhaled corticosteroid $\geq 800\mu\text{g}$ BDP equivalent per day). Flow-cytometry was successfully performed on all sputum samples, and differential counts were conducted on 16/18 samples (slides from 2 children in the asthma group were too heavily contaminated with epithelial cells to perform a leucocyte differential).

Identification of airway dendritic cell populations

Sputum cells exhibiting a lineage⁻ HLA-DR⁺ phenotype consistent with identification as DC were detected in all 18 children who managed to provide a sample that contained lower airway plugs (figure 1). The frequency of airway DC detected was significantly increased as a proportion of total sputum cells in children with asthma compared with healthy controls (1.29% vs. 0.67% of total sputum cells; $p=0.02$, figure 2A, table 2). The prevalence of CD11c⁺ conventional DC and CD123⁺ plasmacytoid DC did not differ significantly between groups (figure 2B and 2C). A small proportion of sputum cells falling within the lineage⁻ HLA-DR⁺ gate exhibited a CD11c^{-dim} CD123⁻ phenotype that did not meet our *a priori* definition of DC, and CD11c expression was not determined in one child in the asthma group. Our initial strategy of excluding non-hematopoietic cells based on differential expression of CD45 was found not to influence the identification of Lin⁻ HLA-DR⁺ DC,

hence this approach was subsequently omitted from the experimental protocol. Together, these findings confirmed that airway DC in children display a subset distribution similar to that reported for adults, and exhibit increased frequency in asthmatic individuals, as has also been identified in adult asthma [11, 12].

Reduced CD86 expression by airway DC in paediatric asthma

The proportion of airway DC expressing the co-stimulatory marker CD86 was significantly reduced in children with asthma compared with healthy, age-matched controls (59.7% vs. 73.4%, $p=0.04$, table 2, figure 3A), suggesting that airway DC phenotype is modulated in asthmatic disease and/or is significantly influenced by the effects of concurrent steroid therapy. CD86 expression was not determined in one sample from a child with asthma.

Airway DC in children are predominantly CD1c⁺ cells

The majority of sputum DC displayed phenotypes consistent with identification as CD1c⁺ DC (mean 57% CD1c/B220⁺) or CD141⁺ DC (mean 4.4% CD141/B220⁺), although a substantial proportion of airway DC expressed neither subset marker. There was no significant difference in the prevalence of these populations between children with asthma and healthy controls (table 2, figure 3B, 3C). These data indicated that reduced CD86 expression by airway DC in children with asthma cannot be attributed to changes in DC subset balance.

Airway DC phenotype does not correlate with atopic status, eosinophilia or inhaled steroid dose

Airway DC numbers were similar between children who were atopic ($n=9$) and non-atopic ($n=9$), irrespective of asthma status (1.0 ± 0.2 vs. $0.9\pm0.2\%$, $p=0.59$, Mann-Whitney test). No

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difference between groups was noted for DC phenotype or CD86 expression. Analysis by mean fluorescence intensity specifically for HLA-DR and CD86 gave the same result. Within the asthma group, there were no significant differences in DC subset distribution or activation profile between atopic children and non-atopic children. Airway DC characteristics were also found not to correlate with the proportion of sputum eosinophils as determined by differential count ($r=-0.51$, $p=0.30$), with post-bronchodilator z-score FEV₁ ($r=0.03$, $p=0.93$), or with dose of inhaled corticosteroids ($r=0.00$, $p=1.00$). These data indicated that changes in airway DC frequency and phenotype are not significantly associated with gross measures of asthmatic airway inflammation in children. Two children in the control group had sputum eosinophilia >2.5% on induced sputum noted as an incidental finding, one of these children had documented house dust mite sensitivity.

Discussion

In this study, we report that airway DC can be successfully identified and characterized using flow-cytometry to analyze paediatric sputum samples, and we confirm that in healthy children these cells exhibit a subset distribution resembling that found in adults. We also provide evidence that children with steroid-treated asthma exhibit increased frequency of airway DC with significantly reduced expression of the co-stimulatory marker CD86, suggesting that inhaled steroids may impair DC maturation to reduce inflammatory responses in the lung.

It is currently unclear whether increased frequency of airway DC with decreased expression of CD86 represents a component of disease pathology in asthmatic children or instead reflects the effects of steroid therapy on airway DC maturation and/or trafficking. Both McCarthy *et al* [11] and Dua *et al* [12] reported that airway DC comprise ~0.5% of induced

sputum cells in healthy adults, and that DC numbers were increased in asthmatic individuals, consistent with our own analyses of paediatric sputum samples in the current report. Accumulation of airway DC in the lungs of asthmatic individuals may indicate reduced cell trafficking to the draining lymph nodes, increased infiltration of DC and/or precursor cells into the mucosa, or perhaps reflects local differentiation of DC in response to environmental stimuli. While airway DC numbers in children were broadly comparable with those reported in adults, we also observed a significant decrease in CD86 expression by these cells that was not identified in previous studies of airway DC in adult asthma. It is possible that decreased DC expression of CD86 is due to the effects of inhaled steroid use by 6 of the 8 asthmatic children included in our study, since the adult patients enrolled in previous studies of airway DC were not receiving steroids at the time of sputum induction [11, 12]. Indeed, corticosteroids have previously been shown to reduce CD86 expression by blood DC stimulated with lipopolysaccharide, CD40L or TNF α [23, 24], and may also restrict CD86 expression by DC in the lung and/or modulate trafficking of these cells to the regional lymph nodes. Consistent with this concept, previous studies have identified that inhaled corticosteroids reduce numbers of putative DC in endobronchial biopsies from adults with asthma [25], and recruitment of CD1a⁺ DC to the nasal mucosa is inhibited by topical fluticasone treatment in allergic rhinitis [14]. However, it remains possible that reduced DC expression of CD86 in the lungs of asthmatic children is not due to steroid therapy, since we found no association of steroid dose with DC frequency or phenotype.

In the current study we enumerated DC as a proportion of total sputum cells rather than reporting absolute cell frequencies, since there is currently no validated method available to index cell numbers in sputum. Weighing airway plugs or indexing cell numbers to proteins in sputum cannot take into account the ratio of mucus to cells within a plug, and is thus likely to

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be subject to inter- and intra-individual variation. It is therefore possible that our detection of increased proportions of airway DC in asthmatic children was influenced by changes in unmeasured cell populations between groups. Despite this limitation, we observed that airway DC included high numbers of CD1c⁺ cells, which have also been consistently identified in adult lungs, both in whole tissue digests [7] and in IS samples [11]. In contrast, CD141 (BDCA3)-expressing DC were rare in sputum samples, as is also the case in peripheral blood [26]. Similarly, putative plasmacytoid DC (lineage⁻ HLA-DR⁺ CD11c⁻ CD123⁺) were identified in only low numbers in the majority of sputum samples, and were entirely absent in some individuals. Plasmacytoid DC have been reported to induce allergen tolerance in rodent models of allergic airway inflammation [27, 28], and have also been observed to increase in BAL in asthmatic adults after allergen challenge [10]. In children, Upham *et al* demonstrated that pDC numbers in blood are inversely correlated with wheezing, asthma and respiratory infections before the age of 5 [29], and Hagendorens *et al* have shown that blood pDC are reduced in asthmatic and atopic children compared with healthy controls [30]. These studies suggest that pDC may have a significant role to play in asthmatic lung inflammation, although they represented only a trace population among the induced sputum cells analyzed in our study.

Of the 33 sputum samples obtained in our study, only 18 were deemed suitable for processing; a success rate comparable with previous studies using IS in children [19, 20, 31]. Despite the technical limitations of using induced sputum to assess the immunology of the human lung, use of alternative sampling methods including bronchoscopy and bronchoalveolar lavage is restricted to a small number of children with severe asthma [32], and can only rarely be conducted in healthy children. Similarly, due to limited colours on the BD LSR-II, we did not include a live-dead stain in our protocol, and used FSC/SSC

properties to exclude dead cells and debris as per the recent report by Dua *et al* [33]. Our children with asthma were all male, this was a chance occurrence; to our knowledge gender differences in airway leucocyte populations have not been described, although we cannot completely exclude this as a confounder. In view of this, we undertook a secondary analysis of our data by gender, which showed that DC numbers were increased in boys ($p=0.04$, Mann-Whitney test), while CD11c number and CD86 expression did not vary by gender. We speculate that this observed difference is biased by the preponderance of male asthmatics. The prevalence of atopy in our control group is similar to those reported in NHANES (34), and a within-group analysis of asthmatic children for effect of atopy on DC parameters showed no effect. We also considered atopy as a separate variable irrespective of asthma status, and observed no difference in DC parameters between atopic and non-atopic children. However, as the numbers in this report are limited, these results should be subject to subsequent validation in a larger, more heterogeneous population before drawing firm conclusions about the role of both gender and atopy on airway DC subsets and function. Our choice of sampling may also influence these data, as IS samples cells from a different lung compartment in comparison to BAL [16], which may affect comparisons between studies. Including larger numbers of children with both mild, and more severe asthma, will also serve to inform the apparent discrepancy between the results presented in this report and those in the previous adult studies [11, 12] regarding CD86 expression, as greater numbers of steroid-naïve children with asthma, comparable to those adults enrolled in previous reports, may serve to address the role of inhaled corticosteroids on CD86 expression in the airway. The logistics of the school visits prevented detailed functional studies, but future analyses may exploit cell sorting approaches to assess DC characteristics and stimulatory potential in more depth.

In summary, we report the first detailed analysis of airway DC characteristics in induced sputum samples obtained from children. By using flow-cytometry to analyze small numbers of cells in induced sputum samples, we have been able to identify for the first time that children with asthma exhibit increased numbers of airway DC with reduced expression of the co-stimulatory marker CD86, suggesting that inhaled steroid therapy may impair DC maturation to reduce pro-inflammatory responses in the asthmatic lung. It is now feasible to non-invasively assess the effect of novel asthma therapies targeting airway DC functions in children.

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Figure legends

Figure 1: Identification of airway dendritic cell (DC) populations in induced sputum from children. Viable sputum cells were identified on the basis of size and granularity (region R1), and cells that exhibited a lineage⁻ HLA-DR⁺ phenotype consistent with identification as DC were selected for further analysis (R2). Airway DC expression of the subset markers CD1c and CD141, and co-stimulatory molecule CD86, was determined relative to isotype-matched control mAbs. CD11c expression was determined relative to the fluorescence levels of autologous non-myeloid sputum cells in the same sample. Percentage values in the figure refer to the results for the individual. The CD123+ gate is from a separate child.

Figure 2: Airway DC exhibit increased frequency but normal subset distribution in children with asthma. Frequency of total DC (A) myeloid DC (B) and plasmacytoid DC (C) in healthy children and children with asthma were determined. Bars indicate mean values. Filled circles represent children with atopy. * $p < 0.05$, Student's t-test.

Figure 3: (A) Reduced frequency of CD86 expression by airway DC in paediatric asthma. Airway DC in sputum samples from children with asthma expressed significantly lower levels of the co-stimulatory marker CD86 relative to sputum DC from healthy controls. (B) CD1c/BDCA-1⁺ cells were common among sputum DC in both healthy children and children with asthma ($p = \text{NS}$ between groups), whereas (C) CD141/BDCA-3⁺ cells were rare and displayed a trend towards increased numbers in the asthma group. Bars indicate mean values. Filled circles represent children with atopy. * $p < 0.05$, Student's t-test.

	<i>Controls</i>	<i>Asthma</i>
n	10	8
Age	11.5 (0.45)	11.0 (0.67)
Gender (M/F)	6/4	8/0
Atopy	5/10	4/8
Post bronchodilator z-FEV ₁	-0.27 (0.38)	-0.56 (0.32)
Post bronchodilator z-FVC	-0.80 (0.42)	-0.33 (0.34)
Inhaled corticosteroid dose µg (daily BDP equivalents)	Not applicable	412.5 (142)
Sputum macrophages %	88 (3.9)	86 (5.3)
Sputum neutrophils %	10 (4.1)	13 (5.6)
Sputum eosinophils %	2.2 (1.4)	1.1 (1.1)
Sputum lymphocytes %	0 (0.0)	0 (0.0)

Table 1: participant demographics. Atopic sensitization was determined by skin prick test to common aeroallergens. Data are presented as mean $\pm$ standard error. z-FEV₁; standard deviation scored forced expiratory volume in 1 second. FVC; forced vital capacity. BDP; budesonide dipropionate. Leucocyte differential count data was obtained from 6/8 children in the asthma group.

	<i>Controls</i>	<i>Asthma</i>	<i>p value</i>
Induced sputum DC (% total sputum cells)	0.67 (0.17)	1.29 (0.17)	0.021*
CD11c ⁺ conventional DC (% sputum DC)	69.3 (2.2)	65.6 (4.6)	0.44
CD123 ⁺ plasmacytoid DC (% sputum DC)	1.8 (0.80)	4.4 (1.56)	0.134
CD86 ⁺ DC (% sputum DC)	73.4 (4.0)	59.7 (4.5)	0.039†
CD1c DC (% sputum DC)	56.3 (3.2)	57.0 (3.9)	0.89
CD141 DC (% sputum DC)	2.4 (0.92)	6.9 (2.26)	0.065

Table 2. Dendritic cell populations and co-stimulatory marker expression in healthy controls and children with asthma. Data are presented as mean $\pm$ standard error. DC, dendritic cell; cDC, conventional dendritic cell. *Mann-Whitney test, †unpaired Student's t-test.



