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ORIGINAL PAPER

Inhaled allergen-driven CD1c up-regulation and enhanced antigen uptake by activated human respiratory-tract dendritic cells in atopic asthma

N. E. McCarthy*, H. A. Jones†, N. A. Marks†, R. J. Shiner†, P. W. Ind†, H. O. Al-Hassi*, N. R. English*, C. M. Murray‡, J. R. Lambert§, S. C. Knight* and A. J. Stagg*

**Antigen Presentation Research Group, Imperial College London, Northwick Park & St. Mark's Campus, Middlesex, UK, †Department of Respiratory Medicine, Imperial College London, Hammersmith Campus, London, UK, ‡Discovery Bioscience, AstraZeneca R & D Charnwood, Loughborough, UK and §PAREXEL Clinical Pharmacology Research Unit, Northwick Park Hospital, Middlesex, UK*

Clinical and Experimental Allergy

Summary

Background Dendritic cells (DC) mediate inflammation in rodent models of allergic airway disease, but the role played by human respiratory-tract DC (hRTDC) in atopic asthma remains poorly defined. Recent data suggest that CD1 antigen presentation by hRTDC may contribute to asthma pathogenesis.

Objective To investigate the influence of hRTDC on the balance between atopy and allergic asthma in human subjects and to determine whether CD1 expression by hRTDC is modulated during asthmatic inflammation.

Methods Sputum cells were induced from steroid-naïve, allergen-challenged and allergen-naïve subjects (atopic asthmatics, atopic non-asthmatics and non-atopic controls). hRTDC were identified using monoclonal antibody labelling and analysis by flow cytometry.

Results hRTDC stained HLA-DR$^+$ (negative for markers of other cell lineages) were predominantly myeloid and comprised ~0.5% of viable sputum cells. Sputum cells were potent stimulators of allogeneic CD4$^+$ naïve T cells and enrichment/depletion experiments correlated stimulatory potency with DC numbers. Sputum contained cells that exhibited typical dendritic morphology when analysed by electron microscopy. Myeloid hRTDC were endocytically active, but uptake of FITC-dextran was enhanced in cells from asthmatics ($P < 0.001$). Despite their increased endocytic capacity, asthmatic myeloid hRTDC appeared mature and expressed increased levels of maturation markers ($P < 0.05$–$P < 0.001$), CD1c, CD1d and langerin ($P < 0.05$). CD1c expression by asthmatic myeloid hRTDC was enhanced upon *in vivo* allergen challenge (three to ninefold within 24 h; $P < 0.05$). CD11c$^-$CD123^{high} hRTDC were only detected in asthmatic sputum and were increased in number following allergen challenge.

Conclusion Despite limited cell numbers, it proved possible to analyse human RTDC in induced sputum, providing evidence that increased antigen uptake and enhanced CD1 presentation by activated hRTDC may contribute to allergic airway disease. CD1 presentation by hRTDC in atopic asthma may therefore constitute a novel target for future intervention strategies.

Correspondence:
Andrew J. Stagg, Antigen Presentation Research Group, Imperial College London, Northwick Park & St. Mark's Campus (Level 7W), Watford Road, Middlesex, HA1 3UJ, UK.
E-mail: a.stagg@imperial.ac.uk

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Introduction

Allergic asthma is characterized by chronic eosinophilic inflammation and reversible obstruction of the airways [1]. While up to 50% of the western population is sensitized to allergens, only 7–10% of adults exhibit symptoms of asthma [2]. Additional factors are evidently required to confer asthmatic disease.

Dendritic cells (DC) are the most potent known activators of naïve T cells [3]. DC present peptide–MHC complexes to T cell subsets, but can also present antigens in the context of CD1, a family of molecules that

incorporates multiple different isoforms (CD1a, CD1b, CD1c and CD1d) [4 – 6]. DC mediate the immune response to inhaled allergens and are thought to play a critical role in asthma pathogenesis [7], but detailed analyses of respiratory-tract DC (RTDC) are currently limited to studies in animal systems. Recent data suggest that CD1 antigen presentation by human respiratory-tract dendritic cells (hRTDC) may contribute to asthma pathogenesis [8]. Indeed, in the case of phospholipid antigens extracted from pollen, CD1a and CD1d expressed by monocyte-derived DC are involved in antigen binding and presentation to a number of different αβ and γδ T cell subsets, including natural killer T cells (NKT cells) [9]. These observations lead to the hypothesis that hRTDC expression of CD1 molecules is altered in asthma and contributes to disease pathogenesis by stimulating CD1-restricted lymphocytes (such as NKT cells) in the pulmonary tissues [10]. However, it is currently unknown whether CD1 expression by RTDC is modulated in the asthmatic human lung.

In murine models, altering the balance of DC subsets in the lung is sufficient to induce pulmonary eosinophilia [11], while under non-inflammatory conditions, DC can produce regulatory cytokines and mediate allergen tolerance [12]. However, the extent to which data from mouse models of asthma can be extrapolated to the human disease remains unclear [13], and a number of key points arising from murine models remain untested in the human system.

DC are known to reside in the asthmatic lung and increase in number following allergen challenge [14]. However, many early studies of hRTDC used isolation protocols that incorporated culture steps that we now recognize induce phenotypic and functional changes in DC [15, 16]. We have developed a non-invasive approach to the analysis of hRTDC in induced sputum that limits *ex vivo* manipulation, thereby minimizing inadvertent effects upon DC phenotype. Multi-parameter analysis of unmanipulated hRTDC by flow cytometry allows unambiguous identification of DC subsets and quantitative analysis of their phenotype that has not previously been possible using only histological approaches. Indeed, detailed characterization of the predominant DC subset in murine lung has only recently been reported [17], and comparable data for the human airways are not yet available.

Using a novel, multi-parameter approach, we aimed to investigate the influence of hRTDC on the balance between atopy and allergic asthma in human subjects and to determine whether CD1 expression by hRTDC is modulated during asthmatic inflammation. Here, we report the successful characterization of the trace DC populations present in sputum from human subjects and identify changes in DC activation, antigen uptake and expression of CD1c and CD1d that are specifically associated with atopic asthma. Furthermore, we demonstrate that hRTDC expression of co-stimulatory molecules and CD1c antigen-presenting molecules is modulated upon allergen inhalation. These findings support a critical role for $CD1^+$ human RTDC in the pathogenesis of allergic asthma.

Methods

Subjects

Sputum donors (non–atopic control = 12, atopic-asthmatic = 15, atopic non-asthmatic = 6) were light/non-smokers. Smoking was prohibited for $\geqslant 1$ year preceding the study, steroids excluded for $\geqslant 1$ month prior and antihistamines precluded for $\geqslant 24$ h before induction. Volunteers were in good general health and free from respiratory infection for $\geqslant 1$ month before taking part. Written, informed consent was obtained from all participants. The study was approved by the Harrow Research Ethics Committee.

Spirometry

Lung function was measured using routine spirometry (Vitalograph, Bucks or Jaeger-Masterscope, Wurzberg, Germany). Asthmatic subjects presented with mild, reversible airway obstruction and a clinical history of asthma (Table 1).

Skin-prick testing

D. pteronyssinus, dog, cat, feathers, grass pollen, *C. herbarum*, *A. alternata*, *C. albicans*, and *A. fumigatus* (ALK-Abelló, Hørsholm). Atopic donors displayed positive reactions to $\geqslant 2$ aeroallergens or exhibited an atopic family history and 1 positive reaction.

Allergen challenge

The allergen challenge procedure was modified from published guidelines [18]. Asthmatic subjects who exhibited a forced expiratory volume in 1 s (FEV_1) > 70% of predicted values, a positive skin test to Der p 1 and/or Fel d 1 and methacholine PC_{20} < 8 mg/mL were challenged with the corresponding allergen (Aquagen SQ, 250 – 64 000 U/mL; ALK-Abelló). Subjects inhaled five breaths of normal saline (0.9%) from a dosimeter (Mefar, Brescia, Italy) and gave two consistent measurements of FEV_1 at 5 and 10 min post-saline inhalation. Allergen was administered in the same way as the saline with FEV_1 measurements being taken at 5 min intervals. Where FEV_1 fell < 15% from post-saline baseline, the allergen dose was doubled. Where FEV_1 declined 15–20% from post-saline baseline, the allergen dose was unchanged. The challenge procedure continued until a $\geqslant 20\%$ decline in FEV_1 was achieved.

Table 1. Subject demographics

		Age	FEV_1 %Pred*	%Improve†	FEV_1/FVC%‡	%Eos§
Asthma;	*Mean*	35	88.51	9.62	78.46	3.86
	Range	21–67	53.8–115	0–29.73	50–96.8	0.4–18.8
Control;	*Mean*	32	100.36	1.97	84.63	0.1
	Range	21–57	83.7–115	0–6.3	74.3–99	0–0.2
Atopic;	*Mean*	42	108.6	0.44	84.55	0.16
	Range	22–61	85.3–123.6	0–1.4	70.5–92.1	0–0.6

*FEV_1 as a function of predicted values (adjusted for age, sex, weight and ethnicity).
†Post-bronchodilator improvement in FEV_1.
‡FEV_1/FVC ratio.
§Sputum eosinophilia as assessed by differential counting.
FEV_1, forced expiratory volume in 1 s, FVC, forced vital capacity.

Sputum induction

Sputum induction and processing were consistent with published guidelines [19]. Volunteers were pre-treated with 200 μg inhaled salbutamol 15 min before induction. Donors inhaled nebulized saline (4.5%) for 1 min before giving two consistent measurements of FEV_1. Saline was administered via DeVilbiss/Medel (De Vilbiss-Sunrise Medical, Wollaston, UK; Medel–White Medical, Rugby, UK) ultrasonic nebulizers for a maximum of 20 min or until FEV_1 fell $\geqslant$20% from post-bronchodilator baseline. Interruptions occurred at 5 min intervals for measurement of FEV_1 or as sputum was produced. Fluid from the buccal cavity was expectorated into a petri-dish and the mucus plugs selected out.

Sputum processing

Mucus plugs were weighed and digested in a fourfold quantity of 0.1% v/v dithiothreitol (Calbiochem, San Diego, CA, USA). The digestion mixture was incubated at 37 °C for 20 min and then diluted 1:1 with PBS. The resultant cell suspension was filtered through 48 μm gauze and centrifuged (650 *g*, 4 °C, 10 min). The supernatant was discarded and the cells were re-suspended in PBS.

Antibody labelling

Sputum cells were labelled with mAb on ice for 20 min; anti-CD1d (clone CD1d42), β7 Integrin (FIB504), CD80 (L307.4), CD83(HB15e), CD123(9F5), CD34(581) and HLA-DR(G46-6) were from BD Biosciences (Cardiff, UK). Anti-CD3(UCHT1), CD14(RMO52), CD16(3G8), CD19(J4.119), CD124(S456C9), langerin(DCGM4) and CD56(N901) were from Beckman Coulter (High Wycombe, UK). Anti-CD1a (NA1/34), CD40(LOB7/6) and CD86(BU63) were from Serotec (Oxford, UK). Anti-CD1c(AD5-8E7) and BDCA-3(AD5-14H12) were from Miltenyi Biotec (Bisley, UK). Anti-DC-SIGN(120507) was from R&D Systems (Abingdon, UK) and anti-CD11c(KB90) was from Dako Cytomation (Ely, UK). Corresponding isotype-matched controls were purchased from the respective manufacturers. Non-specific binding was blocked by addition of fetal calf serum (FCS) before labelling. The labelled cells were washed in FACS buffer (PBS, 2% FCS, 0.02% sodium azide, 1 mM EDTA) and centrifuged (500 *g*, 4 °C, 5 min). The supernatants were discarded, and the cell pellets fixed in 1% paraformaldehyde.

Flow cytometry

Data acquisition was by a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK), and analysis using WinList software (Verity, Maine). Live cells were analysed on a plot of HLA-DR vs. lineage cocktail staining (lin; mAb labels for CD3, CD14, CD16, CD19, CD34 and CD56 against unwanted cell lineages). DC were identified as $HLA\text{-}DR^+lin^{-/dim}$ cells. The proportion of $label^+$ cells was measured by gating on events that exceeded the fluorescence range of the isotype control. Enhanced normalized subtraction was used to measure the staining intensity of the positive cells (expressed as the positive intensity/PI ratio relative to the isotype control).

Endocytosis assay

Sputum cells in complete medium (Dutch-modified RPMI-1640, 10% FCS, 100 μM/Lu penicillin, 100 μgμ ml^{-1} streptomycin, 20 mM L-glutamine) were incubated at 4 °C or 37 °C ml^{-1} with or without 1 mg/mL FITC-dextran for 15, 30, 45 or 60 min. Endocytosis was halted by addition of ice-cold FACS buffer after incubation. Sputum cells were washed in FACS buffer (5 min, 500 *g*, 4 °C), labelled with mAb on ice for 20 min, washed and fixed. Sputum macrophages (autofluorescent $CD14^+CD16^{dim}/lin^{bright}HLA\text{-}DR^+$ cells) were analysed alongside hRTDC as an endocytosis positive control. FITC staining at 4 °C was subtracted from that measured at 37 °C to obtain net mean fluorescence intensity.

Mixed leucocyte reaction (MLR)

Human whole blood was diluted 2:1 with Dutch-modified RPMI-1640 before centrifugation over Ficoll-Paque (Amersham Bioscience) at 650 *g* for 20 min. Peripheral blood mononuclear cells (PBMC) were harvested from the interface and washed in complete medium. $CD4^+$ naïve T cells were negatively selected from PBMC using StemSep (StemCell Technologies, Vancouver, Canada) and separated on Mini-MACS columns (Miltenyi Biotec). The resultant cells were labelled with 5 μM CFSE (Invitrogen, Paisley, UK) for 3 min at room temperature, and the reaction was stopped by addition of FCS.

Unfractionated sputum cells, and sputum cells enriched for or depleted of DC, were used as stimulator cells in an allogeneic MLR with CFSE-labelled, $CD4^+$naïve T cells. Sputum cells were depleted of DC by cell sorting (gated on $CD11c^+lin^-$ cells), or by immunomagnetic separation of $langerin^+$ mononuclear cells. Sorting; sputum cells were labelled with mAb in PBS containing 0.5% BSA and 2 mM EDTA and sorted in exclusion mode on a FACSCalibur flow cytometer. Immunomagnetic separation; sputum cells were centrifuged over Ficoll-Paque (650 *g*, 20 min). The mononuclear cell-rich interface was labelled with mouse anti-human langerin-PE (20 min, 4 °C), washed in FACS buffer and then incubated with anti-mouse IgG microbeads and anti-PE microbeads for 20 min on ice (Miltenyi Biotec). After washing, sputum cells were depleted of $langerin^+$ cells on Mini-MACS columns (Miltenyi Biotec). $Langerin^+$ cells were eluted from the columns as enriched DC.

Enriched blood DC were obtained from the non-adherent fraction of PBMC cultured overnight (37 °C, 5% CO_2) in complete medium at $\sim 4 \times 10^6$ cells/mL. The non-adherent cells were centrifuged over NycoPrep (10 min, 500 *g*; Axis-shield, Oslo, Norway) and the low-density cells harvested from the interface. Responder cells were plated out in 96-well flat-bottom plates at ~400 000 cells per well (cpw). The stimulator cells were added at ~8000 cpw. After 5 days of culture, the cells were harvested and analysed by flow cytometry. Proliferating cells were identified by reduction in CFSE staining, and their number was determined by reference to FlowCount beads (Beckman Coulter).

Electron microscopy

Sputum cells were immuno-gold labelled against HLA-DR (L243, BD Biosciences; goat anti-biotin 10 nm gold, BB International), fixed in 3% glutaraldehyde, embedded in low gelling temperature agarose (Sigma-Aldrich, Poole, UK), further fixed in 1% osmium tetroxide and then stained with 2% uranyl acetate. The cells were dehydrated using an acetone gradient, embedded in araldite resin and cured for 18 h at 65 °C. Blocks of cells were cut into ~100 nm sections and collected onto copper grids. Sections were stained with Reynold's lead citrate and viewed on a Jeol JEM-1200EX electron microscope (Jeol, Welwyn Garden City, UK).

Statistics

Statistical analyses were performed using SigmaStat software (SPSS, Chicago, IL, USA). Pooled data are expressed as mean values ± standard error. *t*-tests were used to analyse normally distributed data and Mann–Whitney rank-sum tests were used to analyse non-normally distributed data. Two-way analysis of variance was used to test the endocytosis data. $P < 0.05$ was considered to be significant.

Results

Identification of hRTDC in sputum

Cells exhibiting a characteristic DC phenotype ($HLA\text{-}DR^+lineage^{-/dim}$) were detected in sputum by flow cytometry (Fig. 1a– R1). The size and granularity of this population were typical of DC (Fig. 1b– R2; black events). hRTDC stained predominantly $CD11c^+$ (Fig. 1c– R3), and their identity was confirmed by immuno-gold electron microscopy (Fig. 1d).

hRTDC comprised ~0.5% of viable sputum cells in allergen-naïve subjects and there was a tendency towards elevated *proportions* of hRTDC in asthmatic sputum cells (%hRTDC in sputum cells; control, 0.31 ± 0.06%; atopic, 0.36 ± 0.08%; asthmatic, 0.64 ± 0.15%). The total *number* of hRTDC obtained by sputum induction was significantly higher in asthmatic subjects than in healthy control donors (Fig. 2a; $P < 0.05$). However, when these values were corrected for the mass of sputum plugs obtained, this difference between asthmatic and healthy control donors was no longer statistically significant (hRTDC number; $24 \pm 6 \times 10^3\,g^{-1}$, $n = 12$ asthmatic subjects; vs. $42 \pm 11 \times 10^3\,g^{-1}$, $n = 12$ healthy control donors; $P > 0.05$), suggesting that increased airway secretions in asthma may partially contribute to elevated DC numbers. Putative plasmacytoid/$CD11c^-$ DC comprised less than 30% of hRTDC in each of the subject groups analysed (Fig. 2b), and sputum from atopic subjects contained a significantly lower proportion of these cells than was detected in control subjects ($P < 0.05$).

hRTDC are potent stimulatory cells

Sputum cells stimulated $CD4^+$ naïve T cells to proliferate with a potency comparable with that of mature blood DC (Figs 3a and b). The small number of cells available in sputum prevented DC purification, but partial enrichment/depletion of hRTDC in sputum cells was used to assay their

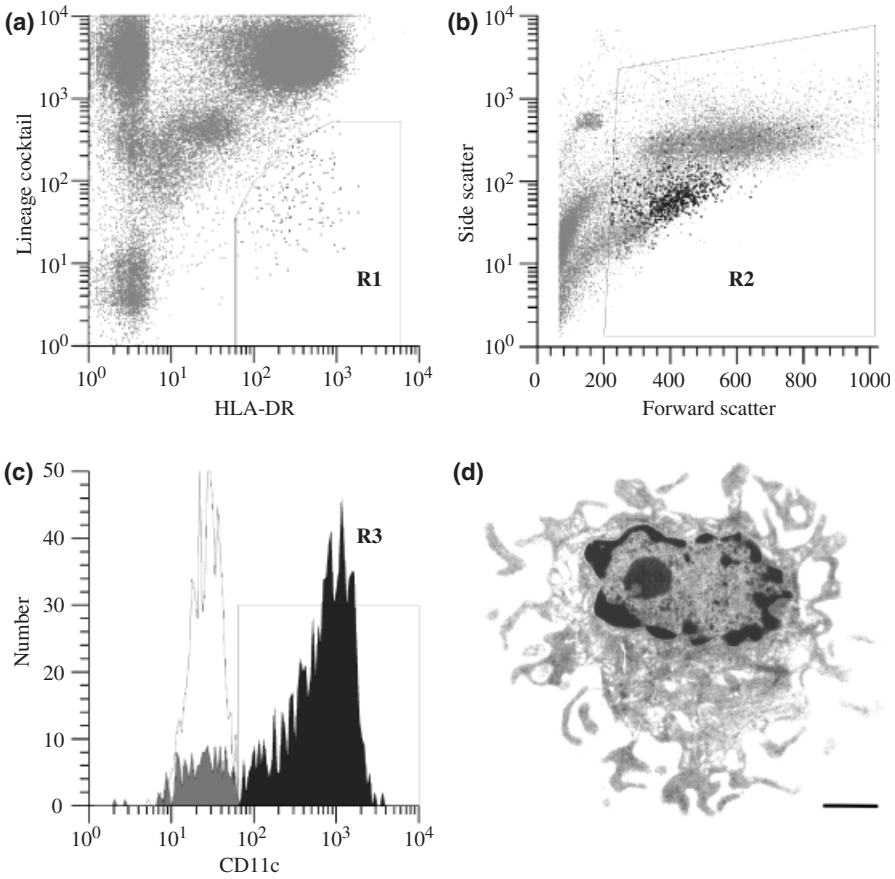

Fig. 1. Human respiratory-tract dendritic cells (hRTDC) can be identified in induced sputum. HLA-DR$^+$ lineage$^{-/dim}$ hRTDC (a – R1) exhibited size and granularity typical of dendritic cells (b – R2; black events). hRTDC stained predominantly CD11c$^+$ relative to isotype-matched controls (c – R3), and their identity was confirmed by immuno-gold electron microscopy (d; HLA-DR$^+$ cells exhibiting dendritic morphology, scale bar = 1 μm).

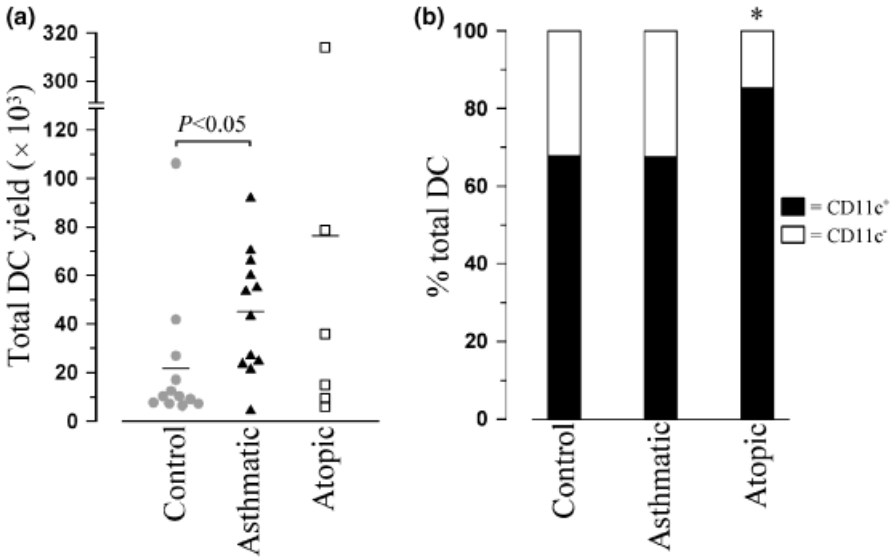

Fig. 2. Higher yields of human respiratory-tract dendritic cells (hRTDC) can be obtained from asthmatic subjects than from healthy controls (a; $P < 0.05$). hRTDC were predominantly myeloid/CD11c$^+$ in each of the subject groups analysed, but the proportion of myeloid DC was increased in atopic sputum when compared with control samples (b; $P < 0.05$).

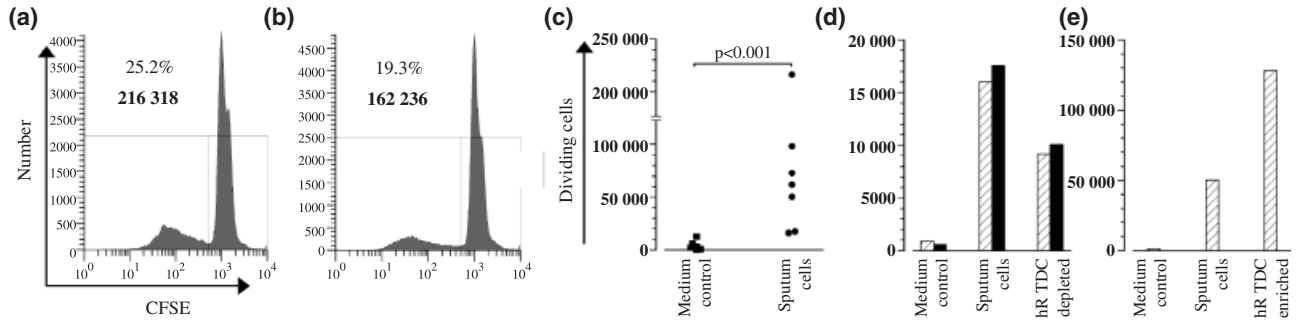

Fig. 3. Sputum cells are potent stimulators of $CD4^+$ naïve T cell proliferation. Sputum cells stimulus (a), blood DC stimulus (b); %, proportion of dividing cells; bold, absolute numbers. Sputum cells stimulated significant T cell proliferation (c). Depletion of human respiratory-tract dendritic cells (hRTDC) from sputum cells by cell-sorting (hatched bars), or column separation (solid bars), reduced stimulatory potency (d). hRTDC enrichment increased stimulation (e).

contribution to the stimulatory effects observed. Sputum cells stimulated significant levels of naïve T cell proliferation (Fig. 3c). However, partial depletion of myeloid hRTDC from sputum cells by cell sorting resulted in a 43% reduction in the number of dividing cells detected (Fig. 3d). A similar reduction was achieved by depletion of langerin$^+$ cells (putative $CD11c^+$ hRTDC) using column fractionation. In contrast, enriching for this langerin$^+$ subset increased T cell proliferation ~2.5-fold compared with the unseparated sputum cells stimulus (Fig. 3e).

Enhanced antigen uptake by asthmatic hRTDC

Endocytosis by $CD11c^+$ hRTDC was rapid and sustained, but asthmatic cells displayed greater activity than their control or atopic counterparts (Fig. 4; $P < 0.001$). Plasmacytoid DC displayed minimal endocytic activity. In contrast, HLA-DR^+lin^+ monocyte/macrophages (Mo/MΦ) displayed ~36-fold higher levels of uptake than $CD11c^+$ hRTDC. While Mo/MΦ from control donors were the most endocytic of the subject groups ($P < 0.001$ compared with asthmatic or atopic Mo/MΦ), hRTDC from these subjects were the least effective at antigen uptake.

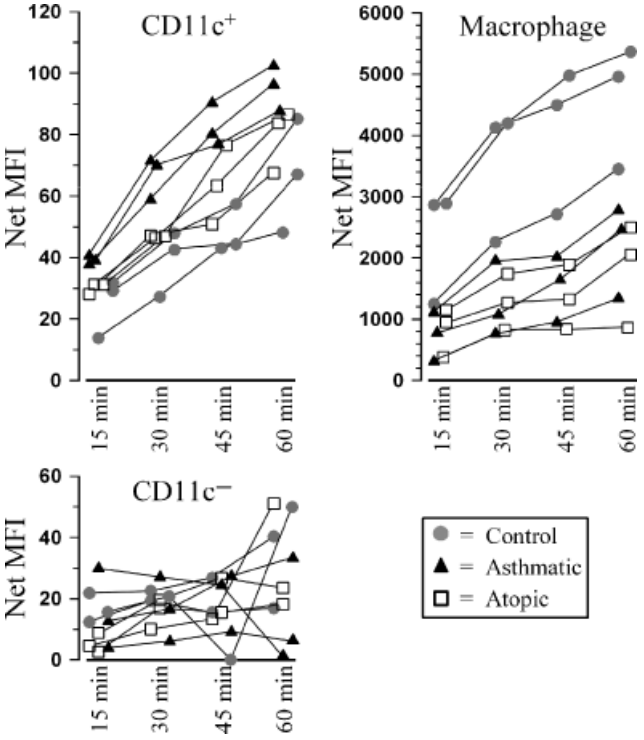

Fig. 4. Enhanced antigen uptake by myeloid human respiratory-tract dendritic cells (hRTDC) in asthma. $CD11c^+$ hRTDC demonstrated rapid and prolonged endocytic activity, with greater efficiency evident in cells from asthmatic donors ($P < 0.001$ compared with control or atopic subjects). $CD11c^-$ putative hRTDC demonstrated only low levels of endocytosis. In contrast, sputum macrophages exhibited far greater levels of endocytic activity than either subset of hRTDC. (MFI; mean fluorescence intensity).

Elevated expression of CD1c, CD1d, langerin and maturation markers by $CD11c^+$ hRTDC in asthma

hRTDC were labelled with mAb against various DC subsetting and maturation markers. The *proportion* of positive cells (%) and the expression *level* of each marker (positive intensity/PI ratio) were determined relative to isotype-matched controls.

Myeloid hRTDC from control subjects expressed only low levels of co-stimulatory molecules. In contrast, hRTDC from asthmatics exhibited a phenotype suggestive of mature DC; they expressed significantly higher levels of CD80 (PI: 11 ± 2) and CD86 (PI: 14 ± 3) than hRTDC from controls (PI: 4 ± 1 for CD80; 5 ± 1 for CD86 – Fig. 5a). The proportion of hRTDC expressing CD80 or CD86 in asthmatic sputum ($50 \pm 5\%$ and $71 \pm 4\%$, respectively) was also significantly higher than was detected in sputum from control (CD80, $23 \pm 6\%$; CD86, $38 \pm 6\%$) or atopic subjects (CD80; $24 \pm 7\%$; CD86, $41 \pm 10\%$ – Fig. 5b).

Changes in hRTDC phenotype associated with asthma were not confined to activation markers alone. Asthmatic $CD11c^+$ hRTDC expressed significantly higher levels of CD1c, CD1d, CD123 and langerin than equivalent cells

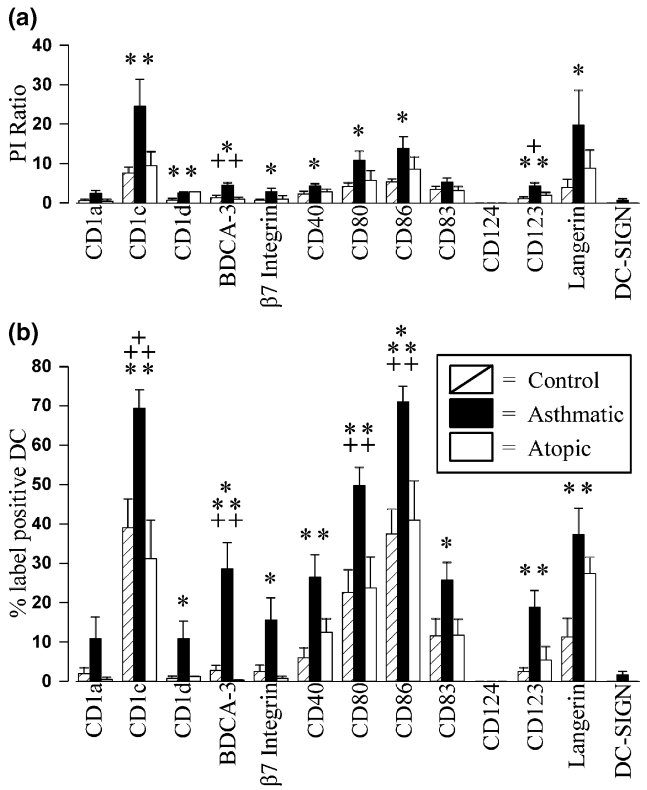

Fig. 5. Asthmatic CD11c$^+$ human respiratory-tract dendritic cells (hRTDC) exhibit a CD1$^+$ mature phenotype. The staining intensity (a) and proportion of positive cells (b) labelled with specific mAb were measured relative to isotype-matched controls. Bars indicate mean values+standard error. $^*P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{***}P \leqslant 0.001$ vs. control, $^+P < 0.05$ vs. atopic, $^{++}P < 0.01$ vs. atopic, $^{+++}P \leqslant 0.001$ vs. atopic. Atopic asthmatic, $n = 12$; non-atopic control, $n = 12$; atopic non-asthmatic, $n = 6$.

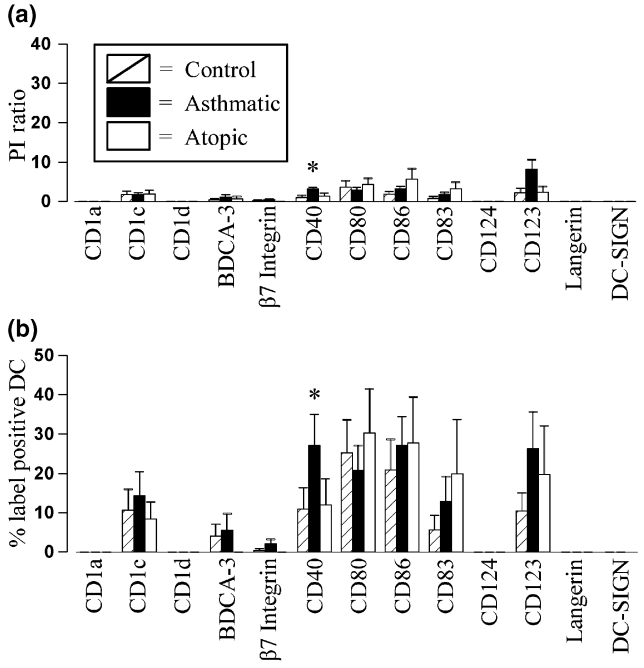

Fig. 6. CD11c$^-$ human respiratory-tract dendritic cells (hRTDC) display increased CD40 expression in asthma. The staining intensity (a) and proportion of positive cells (b) labelled with specific mAb were measured relative to isotype-matched controls. Bars indicate mean values+standard error. $^*P < 0.05$ vs. control. Atopic asthmatic, $n = 12$; non-atopic control, $n = 12$; atopic non-asthmatic, $n = 6$.

from control subjects (Fig. 5a). The *proportion* of CD11c$^+$ hRTDC expressing CD1c was also significantly increased (asthmatic, $70 \pm 5\%$; control, $39 \pm 7\%$), and whereas few CD1d$^+$ cells were detected among the hRTDC population of control or atopic donors ($< 2\%$ stained CD1d$^+$ in these subject groups), there was a significant number of CD1d$^+$ DC in sputum from asthmatics ($11 \pm 4\%$ - Fig. 5b).

CD11c$^-$ 'hRTDC' from asthmatic donors include CD123^{high} cells and display elevated CD40 expression

CD11c$^-$ 'hRTDC' stained only weakly with most of the reagents tested. It is therefore unclear whether these cells correspond to conventional CD11c$^-$ plasmacytoid DC. Cells expressing high levels of CD123 (a hallmark of plasmacytoid DC in peripheral blood) were only detected in sputum from asthmatic donors, and then only in small numbers. Despite their indeterminate origins, CD40 expression was both increased on these cells and detected on a greater proportion of their population in sputum from asthmatics (Fig. 6).

In a subset of donors, sputum was obtained on two separate occasions approximately one week apart ($n = 3$ per allergen-naïve subject group). Surface labelling analyses were performed on both occasions to assess phenotype stability over time. Despite diurnal variability in the disease symptoms of asthmatic subjects, CD11c$^+$ hRTDC exhibited a remarkably consistent phenotype in each of the subject groups analysed (Fig. 7).

Allergen challenge enhances CD1c expression by hRTDC

In a subset of asthmatics, sputum was obtained on three separate occasions over the course of an allergen challenge. hRTDC phenotype was analysed pre-challenge, 24 h post and 72 h post-challenge. The pre-challenge and 24 h post-challenge analyses are shown in Fig. 8.

hRTDC from asthmatic subjects comprised ~0.5% of viable sputum cells before allergen administration, but by 24 h post-challenge the proportion of hRTDC had increased between 1.6-and 9.5-fold. The proportion of hRTDC expressing CD11c also increased markedly following allergen inhalation ($54 \pm 10\%$ in allergen-naïve sputum, $82 \pm 4\%$ in post-challenge samples, data not shown).

Expression of CD1c by asthmatic myeloid hRTDC was increased between 3- and 9-fold within 24 h of allergen administration ($P < 0.05$; Fig. 8). In two of three donors,

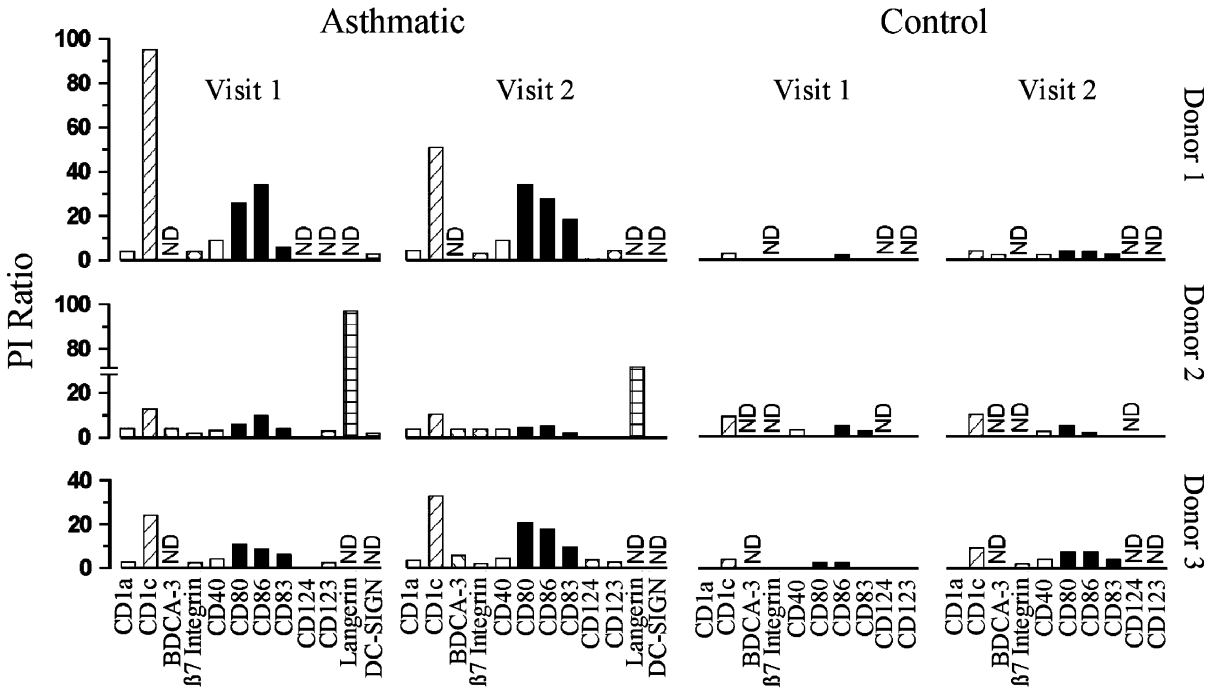

Fig. 7. CD11c$^+$ human respiratory-tract dendritic cells (hRTDC) exhibit a stable phenotype. The staining intensity of hRTDC labelled with specific mAb was measured on two separate occasions approximately one week apart (relative to isotype-matched controls) in three individual subjects per allergen-naïve subject group. The phenotype of atopic CD11c$^+$ hRTDC was indistinguishable from the control phenotype by this analysis. ND = not determined.

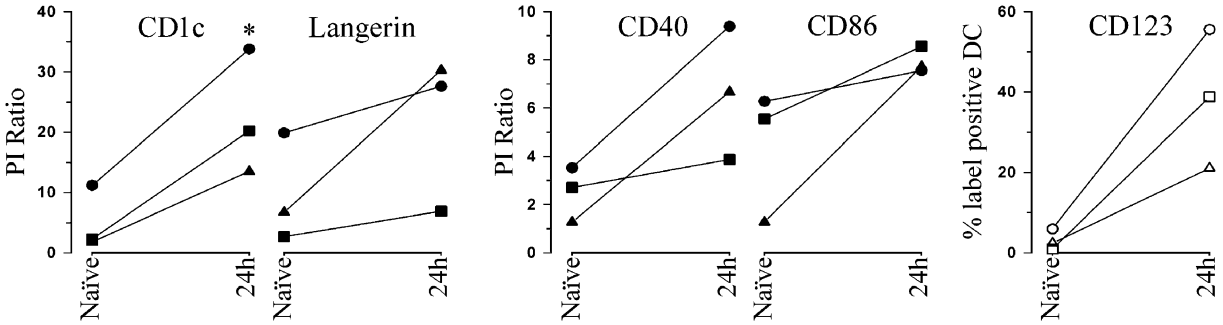

Fig. 8. Asthmatic human respiratory-tract dendritic cell (hRTDC) phenotype is modulated upon allergen challenge. Asthmatic hRTDC were analysed pre- and 24 h–post-allergen challenge; subject 1, •; subject 2, ▲; subject 3, ■. Solid symbols = expression level of respective markers on myeloid hRTDC relative to isotype-matched controls. Open symbols = proportion of CD123$^+$ plasmacytoid DC detected. $^*P < 0.05$ vs. allergen-naïve phenotype.

CD1c expression declined to an intermediate level at 72 h post-challenge (between 1.9- and 3.6-fold of allergen-naïve baseline) but in the third subject, CD1c expression was increased yet further (~19-fold of allergen-naïve baseline, data not shown). Langerin expression by asthmatic myeloid hRTDC was also augmented following allergen inhalation; 24 h post-challenge, levels of staining had increased to more than double those observed on autologous, allergen-naïve cells. CD40 and CD86 expression by CD11c$^+$ hRTDC from asthmatic subjects were also enhanced upon allergen challenge – the intensity of staining for these co-stimulatory molecules approximately doubled on pre-challenge levels. In addition, allergen challenge increased the proportion of CD11c$^-$ CD123^{high} hRTDC detected in asthmatic sputum (Fig. 8), although detected no other marked effects upon the CD11c$^-$ population were detected.

Discussion

hRTDC were successfully identified in induced sputum using multi-colour flow cytometry. This non-invasive approach revealed that hRTDC activation, increased capacity for antigen uptake and enhanced expression of CD1c and CD1d are specifically associated with atopic asthma.

hRTDC displayed a typical DC phenotype, morphology and functional characteristics. Sputum cells stimulated the proliferation of $CD4^+$ naïve T cells with a potency comparable with that of mature blood DC. Although purification was prohibited by low cell numbers, enrichment/depletion of DC in sputum cells identified hRTDC as the major stimulatory cells present. Consistent with these data, $CD1a^+$ DC from bronchoalveolar lavage fluid are reported to be potent accessory cells [20], whereas alveolar macrophages are defective in this role [21].

hRTDC comprised $< 0.4\%$ of viable sputum cells from control or atopic subjects, but $\sim 0.6\%$ of viable sputum cells from asthmatic subjects. Small numbers of $CD123^{high}CD11c^-$ hRTDC were consistently detected, but only in asthmatic sputum. Moreover, allergen inhalation increased the number of $CD123^{high}CD11c^-$ hRTDC observed in asthmatic sputum, alongside increases in the number of myeloid hRTDC detected – both in direct correlation with airflow obstruction. These data suggest recruitment of circulating DC precursors to the inflamed asthmatic mucosa [14], and support a role for $CD11c^+$ RTDC as pro-inflammatory mediators in allergic airway disease [11]. In a murine model of asthma, plasmacytoid DC mediate allergen tolerance in the thoracic lymph nodes by inhibiting the pro-inflammatory effects of myeloid RTDC [22]. However, it is unclear whether the $CD123^{high}CD11c^-$ hRTDC described by the current study correspond to the 'tolerogenic' murine population reported by other investigators, or whether these cells are similarly capable of suppressing inflammation in the human lung.

The conventional model of DC maturation associates enhanced stimulatory potency with decreased endocytic capacity [3]. However, despite the increased maturity of myeloid hRTDC in asthma, these cells were more endocytic than their control or atopic counterparts and expressed high levels of langerin, a receptor for antigen internalization [23]. Allergen challenge further increased langerin expression by asthmatic myeloid hRTDC, despite a concomitant increase in the expression of maturation markers. These data indicate that the regulation of stimulatory capacity and antigen acquisition can be uncoupled in the airways. In asthma, this dichotomy of function may permit continuous sampling and presentation of respiratory antigen by mature, stimulatory hRTDC. Indeed, the reduced endocytic activity displayed by sputum Mo/MΦ in asthma could contribute to this effect by allowing respiratory antigen to accumulate in the airways and increase hRTDC exposure to maturational stimuli.

hRTDC expression of langerin, CD1c and CD1d was significantly increased in asthma. $CD1c^+$ DC have been described at sites of airway inflammation by other investigators [6, 14], but our data extend these findings in several ways. Firstly, we have been able to quantify asthma-related changes in the hRTDC phenotype at the single-cell level, revealing that local up-regulation of CD1c expression, as well as enhanced recruitment, can underlie allergen-driven increases in $CD1c^+$ hRTDC in the asthmatic lung [14]. Secondly, we observed heterogeneity within the hRTDC population; both $CD1c^+$ and $CD1c^-$ DC subsets displayed evidence of phenotypic modulation in asthma that was independent of changes in population size. Other investigators have previously identified potent stimulatory subsets of $CD1a^+$, $CD1c^+$ and $BDCA\text{-}3^+$ DC in human lung tissue, as well as a trace population of plasmacytoid DC that displayed only low levels of HLA-DR and co-stimulatory molecules [24]. The data presented in the current study are consistent with these findings, but extend the analysis of hRTDC populations to include quantitative determination of their phenotypic and endocytic modulation in asthmatic disease. It is unlikely that the DC populations we identified in sputum are a contaminating population derived from the blood since they lack the major subset of $CD11c^-$ DC ($BDCA\text{-}2^+$ $BDCA\text{-}4^+$ $CD123^{hi}$) that comprise approximately half of circulating blood DC [25]. Although the anatomical origin of DC obtained by sputum induction remains to be definitively identified, it is likely that many of these cells are derived from the tissues of the conducting airways as analysis using radiolabelled bolus delivery suggests that this is the major site sampled by the induced sputum technique [26]. The similarities between the data presented here and those reported by Demedts et al. [24], in their study of biopsy-derived DC, and more specifically the presence of $CD1a^+$ DC in our samples (cells that are present in the epithelium of the conducting airways but not the lung parenchyma), strongly support the concept that the mucosa of the conducting airways contributes DC to the populations we have analysed. The relative contributions of lumenal, epithelial and submucosal DC populations are harder to determine and may be influenced by the ability of hypertonic saline to increase sampling of the deeper tissues.

While up-regulation of co-stimulatory molecules could simply be indicative of inflammatory activity, this does not provide an adequate explanation for changes in CD1c, CD1d and langerin expression by $CD11c^+$ hRTDC. Indeed, langerin can mediate the uptake of exogenous lipid antigen for CD1 presentation by DC [27], and the activation of 'unconventional' T cells by this route could play a critical role in asthma pathogenesis [8].

$\gamma\delta$ T cells and $CD4^-CD8^-$ $\alpha\beta$ T cells can be activated by respiratory antigen presented in the context of CD1 [28], and CD1d-restricted NKT cells could play a critical role in asthmatic disease [10], but it is unclear whether DC presentation of CD1-restricted antigens is modulated in the asthmatic respiratory tract. CD1 molecules mediate the presentation of ubiquitous environmental antigens of both microbial and self origin [29, 30]. Cross-talk between DC and CD1-restricted T cells is bi-directional and can induce maturation of $CD1^+$ immature, myeloid DC that

present appropriate self-antigen [6, 31]. There is also evidence that RTDC maturation can be induced by interaction with T cell subsets in the airway mucosa [32]. It is possible then that enhanced recognition of CD1 : self-antigen complexes in the pulmonary tissues leads to inappropriate hRTDC maturation by innate lymphocytes such as NKT cells [10]. Increased expression of co-stimulatory molecules could subsequently enhance hRTDC presentation of MHC/CD1–antigen complexes. Similar events have already been described *in vitro*; maturation of $CD1c^+$ myeloid DC – an abundant population in asthmatic sputum – can be induced by cell : cell contact with CD1c-restricted γδ T cells [6]. The resultant mature, $CD1c^+$ DC are endocytic, can present novel protein antigen and are potent stimulators of naïve T cells, features identified as characterizing asthmatic, myeloid hRTDC in the current study.

$V\delta1^+$ γδ T cells constitute the main tissue subset of γδ T cells and recognize CD1c in the absence of foreign antigen [6]. These cells are predominantly auto-reactive and may facilitate rapid immune activation in the peripheral tissues. $V\delta1^+$ γδ T cells are enriched in the lungs of asthma patients following disease exacerbations and produce TNF-α upon mitogen stimulation [33]. γδ T cells play an important role in pulmonary homeostasis [34], and can produce TNF-α to induce maturation of $CD1c^+$ myeloid DC [6, 35]. TNF-α has been implicated in numerous models of DC maturation by innate lymphocytes [36], and may depend upon up-regulated ICAM-1 expression and enhanced lymphocyte clustering [35]. It is intriguing therefore that TNF-α production is increased in the asthmatic lung [37], and that allergen exposure induces ICAM-1 up-regulation and *in situ* maturation of pulmonary DC in a murine model of asthma [32, 38].

Pollen contains bioactive lipids that can modulate DC function [39]. Pollen-derived lipids can also be presented by $CD1^+$ DC *in vitro* [9], and are attractive candidates for CD1 presentation by hRTDC in the airways. In the current study, allergen inhalation enhanced CD1c expression by asthmatic myeloid hRTDC between 3- and 19-fold vs. the allergen-naïve phenotype. It is possible then that regulation of CD1 expression by hRTDC plays a critical role in allergic asthma, influencing DC maturation by innate lymphocytes and presentation of pollen-lipid antigens. Moreover, it is tempting to speculate that the ability of mycobacteria to inhibit CD1 expression by DC [40] may underpin the protection against asthma conferred by childhood exposure to mycobacterial products [41].

Airway DC have been extensively studied in animal systems, but parallel work in the human system has lagged behind. The heterogeneity of hRTDC, when coupled with the diversity of methods, cell sources and animal models used to study these cells, probably accounts for the discrepancies observed in earlier data. The novel approach used by the current study limits the effects of *ex vivo* manipulation on RTDC phenotype [15, 16], and avoids contamination with blood and peripheral lung DC – populations that are distinct from mucosal hRTDC [32, 42].

Currently, we can only speculate upon the possible reasons for the differential phenotype and function exhibited by myeloid hRTDC in asthma, but the data reported here are striking and may prove critical in asthma pathogenesis. Further definition of aberrant hRTDC function in allergic asthma will further our understanding of immune regulation in the human airway and may lead to the identification of novel targets for therapeutic interventions.

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