

Rapid response of circulating myeloid dendritic cells to inhaled allergen in asthmatic subjects

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Summary

Background Dendritic cells (DC) are thought to play a key role in the initiation and maintenance of T cell immunity to inhaled antigens. While the density of DC within the bronchial mucosa is increased in stable asthma, there is little information currently available concerning the effects of allergen inhalation on DC in subjects with asthma.

Objectives To enumerate changes in the numbers of circulating CD33⁺ myeloid DC in asthmatics, before and after allergen challenge.

Methods Blood DC numbers were enumerated by flow cytometry before and at 3, 6 and 24 h after inhaled allergen and diluent in 10 mild, allergic asthmatic subjects.

Results Blood DC numbers rapidly fell from $3.42 \pm 0.30 \times 10^7/L$ at baseline, to $2.10 \pm 0.17 \times 10^7/L$ by 3 h post-challenge ($P < 0.01$), and remained significantly below baseline values at both 6 and 24 h following allergen challenge. No such changes in DC numbers were noted after diluent challenge. A similar, early fall in circulating lymphocytes was also noted post-allergen challenge, whereas changes in circulating eosinophil and neutrophil numbers occurred more slowly.

Conclusions A significant proportion of myeloid DC rapidly ‘disappear’ from the circulation following allergen inhalation, suggesting that margination of circulating myeloid DC, and their recruitment into the airway mucosa, is an important feature of the immune response to inhaled allergen.

Keywords allergens, asthma, cell trafficking, dendritic cells

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Introduction

Allergic asthma is characterized by airway inflammation, in which eosinophils and T cells infiltrate the airways. However, T cells are unable to respond to antigen independently of antigen presenting cells (APC). Because dendritic cells (DC) are the most potent APC within the lung, there is much interest in the role that DC might play in the initiation and amplification of T cell-dependent immune responses to inhaled allergens.

By virtue of their location within the airway epithelium, DC are ideally situated to sample inhaled antigens, and together with their potent capacity for antigen uptake, DC are able to function as sentinels of the immune system. Following their migration to regional lymph nodes, DC undergo functional maturation, developing the requisite cellular machinery necessary to activate resting T cells and initiate immune responses [1, 2]. Studies in experimental animals indicate that airway DC are a highly dynamic population that can rapidly increase in number following local inflammatory stimuli. Both inoculation

with live bacteria and inhalation of allergen induce DC recruitment into the airway epithelium, though allergen is only able to achieve this effect in previously sensitized animals [3, 4].

The limited available evidence indicates that lung DC numbers are also increased in humans, in the context of tissue inflammation associated with cigarette smoking and stable asthma [5–7], although is not clear whether this is due to enhanced DC recruitment from the circulation, to enhanced differentiation from local precursor populations, or to impaired DC migration to regional lymph nodes. In order to examine the first of these mechanisms (i.e. recruitment of DC from the circulation), we studied circulating myeloid DC in subjects with asthma undergoing allergen inhalation challenge, employing a recently developed method to enumerate circulating DC numbers [8]. It was thus possible to delineate the DC response to allergen inhalation at multiple time-points, and to demonstrate that allergen challenge induces a rapid fall in the number of circulating myeloid DC in subjects with asthma.

Methods

Subjects

Ten non-smoking subjects with mild asthma were recruited for the study. The subjects consisted of four females and six males, with an age range of 20–37 years. All subjects had clinically

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stable asthma, with a mean methacholine provocative concentration causing a 20% fall in FEV₁ (PC₂₀) of 2.06 mg/mL (GSEM 1.97), and with no history of worsening symptoms, respiratory tract infection or overt exposure to sensitizing allergens within the preceding 4 weeks. All subjects had an FEV₁ exceeding 70% of the predicted value prior to inhalation challenge with allergen or diluent. Subjects used no medications other than occasional (less than daily) inhaled β_2 -agonist. All medications were withheld for at least 8 h before each visit, and subjects were instructed to refrain from vigorous exercise and caffeine-containing beverages in the morning before visits to the laboratory. The study was approved by McMaster University Health Sciences Centre's Ethics Committee, and subjects gave written informed consent before beginning the study.

Study design

The subjects attended the laboratory on 5 days. The first day was a screening visit. On the second and fourth days, an allergen or diluent inhalation challenge was performed. The third and fifth days were the day following the challenge. Days two and four were separated by at least 2 weeks.

Screening visit

During the screening visit, spirometry was measured with a Collins water-sealed spirometer and kymograph, and allergen skin prick testing was performed against a panel of common aeroallergens. Methacholine inhalation challenge was performed as described by Cockcroft [9]. The test was terminated when the FEV₁ had declined by at least 20% of the baseline value, and the PC₂₀ was calculated by linear interpolation.

Allergen inhalation challenge

At least 24 h after the screening visit, subjects underwent an inhalation challenge with either allergen or diluent, as described elsewhere [10]. The allergen extract selected for inhalation was that which had produced the largest skin test wheal. Thus, six subjects were challenged with ragweed, while three subjects were challenged with house dust mite extract. Starting concentrations of allergen for inhalation challenge were individually determined, based on skin test results and methacholine PC₂₀ [11]. Thereafter, doubling concentrations of allergen were administered until a 15% early fall in FEV₁ was reached. The FEV₁ was measured at 10, 20, 30, 45, 60, 90 and 120 min after allergen inhalation, followed by hourly measurements until 7 h after allergen inhalation. Subjects returned to the laboratory 24 h after challenge for a final measure of FEV₁. Six subjects also underwent diluent challenge, employing a similar protocol to the allergen challenge, except that 0.9% saline was inhaled, rather than allergen.

Blood sampling and processing

Venous blood was collected into two 2.5 mL EDTA Vacutainer tubes (Becton Dickinson Instrument Systems, BDIS, Mississauga, Canada) immediately prior to allergen or diluent challenge, and again at 3, 6 and 24 h post-challenge. One of the blood tubes was immediately sent to the clinical haematology laboratory, McMaster Health Sciences Centre, for determination of total leucocyte and differential white cell counts using an automated blood cell counter (Coulter, Burlington, Ontario, Canada). The remaining blood was used for staining of dendritic cells in lysed whole blood, as described below.

Antibodies

The following directly conjugated mAbs were used: mouse antihuman CD14-FITC, CD16-FITC, CD33-PE, HLA-DR-CyChrome (Pharmingen Canada, Mississauga, Ontario, Canada), and the relevant isotype controls mouse IgG₁-FITC, IgG_{2a}-FITC, IgG₁-PE (Becton Dickinson Instrument Systems) and IgG_{2a}-CyChrome (Pharmingen).

Immunofluorescent staining

We have recently developed a flow cytometric method that permits the reliable enumeration of myeloid dendritic cells in peripheral blood, without the need for extensive processing of the blood sample [8]. The method is both valid, reliably identifying the major subset of circulating dendritic cells, is highly reproducible, with minimal intrasample variability, and requires only a small volume of blood.

Preliminary experiments indicated that DC numbers in whole blood remain stable for up to 4 h, provided samples are stored at 4 °C. Accordingly, all samples were placed on ice, and processed within 4 h of collection, as described in detail elsewhere [8]. Briefly, 100 μ L of blood was added to each 6-mL polystyrene tube. Red cells were lysed using NH₄Cl-EDTA lysing solution, and remaining leucocytes were stained with the following directly conjugated mAbs: CD14-FITC, CD16-FITC, CD33-PE and HLA-DR-CyChrome. Duplicate tubes were stained with these antibodies for analysis of DC numbers, while identical amounts of appropriate isotype controls were similarly added to control tubes, as described elsewhere [8].

Flow cytometry

Stained and control cell preparations were analysed within 24 h using a FACScan™ flow cytometer equipped with a 488-nm argon laser (Becton Dickinson Instrument Systems) and operated with CellQuest™ software. Each measurement contained 50 000 to 60 000 mononuclear cells. As tubes were stained in duplicate, the resulting dendritic cell counts were typically based on 900 to 1000 dendritic cells, expressed as a proportion of 100 to 120 000 mononuclear cells. Granulocyte data were not saved, in order to keep computer files to a manageable size.

Off-line analysis was subsequently performed with PCLysis software (version 1.1) as obtained from BDIS. A dot plot of forward vs. side scatter was established, and a region (R1) drawn around the lymphocytes and monocytes, excluding any residual data from granulocytes and cellular debris (Fig. 1, left panel). Next, a histogram of CD14⁺CD16⁺ FITC expression was produced using data gated on R1. A second region (R2) was drawn as a histogram region including cells with negative or low CD14/16 expression, thereby excluding the well-defined populations of cells with high CD14/16 expression (Fig. 1, middle panel). The final dot plot of CD33-PE vs. HLA-DR CyChrome was established by combined gating of events using R1 and R2. Within this last dot plot, a third region was drawn around the CD33^{bright}, HLA-DR positive population, termed region R3 (Fig. 1, right panel).

Determination of DC counts

For each tube, the number of cells defined by region R3 was expressed as a percentage of mononuclear cells defined by region R1. The mean of duplicate tubes was calculated, in order to determine the myeloid DC differential count.

Estimates of the absolute numbers of DC in blood were calculated by multiplying this DC differential cell count by the absolute mononuclear cell count, on the assumption that lymphocytes and monocytes (as determined by the haematology cell counter) together constitute the mononuclear fraction (region R1) identified by flow cytometry.

Statistics

Except where indicated, all data are expressed as mean $\pm$ standard error. The effect of allergen or diluent challenge on numbers of circulating DC at various time-points was analysed by analysis of variance for repeated measures (ANOVA), and by *post hoc* Student's paired *t*-test. Resulting *P*-values <0.05 were regarded as significant.

Results

Identification of circulating DC

Previous investigators have purified CD33⁺(bright), CD14⁻, CD16⁻, HLA-DR⁺ cells from peripheral blood by FACS, and

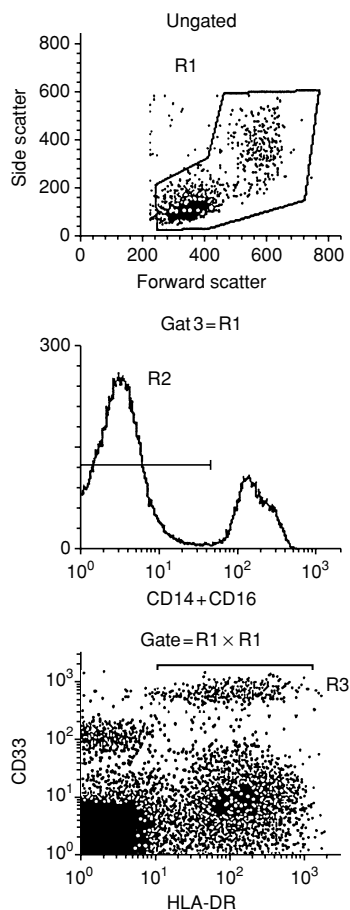


Fig. 1. Identification of circulating dendritic cells by flow cytometry. Dendritic cells were identified in lysed whole blood by three-colour staining with CD14 FITC + CD16 FITC, CD33 PE and HLA-DR CyChrome, employing a sequential gating strategy, as outlined in Methods. DC were thus identified as cells in region 3 (R3), expressing high levels of HLA-DR and CD33, but having little or no expression of CD14 and CD16. The number of DC in R3 was expressed as a percentage of the number of mononuclear cells in region 1 (R1). The mean of duplicate tubes was used to determine the DC differential count.

have shown that these cells possess features typical of DC [12, 13]. In the current study, we identified this same population of cells by immunofluorescence staining and flow cytometric FACS analysis of lysed whole blood (Fig. 1). By first gating out cells that were strongly positive for CD14 and CD16, a discrete cell population was identified that strongly expressed both HLA-DR and the myeloid antigen CD33 (region R3 in Fig. 1). Absolute counts of myeloid dendritic cells were then determined indirectly by multiplying the dendritic cell differential count by the sum of the lymphocyte and monocytes determined on an automated differential blood cell counter. In preliminary experiments we determined that baseline levels of myeloid DC did not differ between normal individuals and those with asthma (data not shown).

Allergen-induced airway responses

The mean ($\pm$ SEM) fall in FEV₁ during the early response was $36.6 \pm 3.4\%$. Seven of the 10 subjects developed a late response (a fall in FEV₁ $> 15\%$ between 3 and 7 h after inhaled allergen). The mean fall in FEV₁ for all subjects during the late response was $18.3 \pm 1.9\%$.

Allergen-induced changes in blood DC numbers

Following allergen challenge, there was a rapid fall in numbers of circulating myeloid dendritic cells in all subjects (Fig. 2), though there was inter-individual variability in the magnitude of this response. The changes were most marked at 3 and 6 hours post-allergen challenge ($P < 0.01$ compared with baseline), but was still evident 24 h after challenge ($P < 0.05$ compared with baseline). These changes were statistically significant regardless of whether the data were expressed as absolute numbers of dendritic cells (as shown in Fig. 2), or as the dendritic cell differential cell count (data not shown). Significant falls in DC numbers were observed in subjects exhibiting a dual asthmatic response, as well as in those exhibiting an isolated early fall in FEV₁. In contrast, diluent challenge had no significant effect on numbers of circulating dendritic cells at 3 h and 6 h (Fig. 3). The effects of allergen challenge on circulating DC numbers did not appear to be due to bronchoconstriction *per se*, as methacholine challenge had no significant effect on blood DC numbers (data not shown).

Changes in other circulating leucocytes

Following allergen challenge, the numbers of circulating lymphocytes fell significantly at 3 h, 6 h and 24 h ($P < 0.05$), which was similar to the dendritic cell response (Fig. 4). In contrast, there was no detectable change in numbers of monocytes at any time-point, regardless of whether this was determined by Coulter counter, or by flow cytometry of CD14⁺ cells (Fig. 4). Finally, eosinophil and neutrophil numbers were significantly below baseline values at the 6 h and 24 h time-points, respectively ($P < 0.05$) (Fig. 4).

Discussion

The novel finding to emerge from this study is that allergen challenge induces a rapid and profound change in the number of circulating myeloid DC in subjects with asthma. Within 3 h of allergen inhalation, and prior to the onset of the late asthmatic response, approximately one-third of myeloid DC had

Fig. 2. Changes in numbers of circulating dendritic cells following allergen inhalation challenge. A marked fall in numbers of circulating myeloid DC was noted at all time-points after allergen challenge. ** $P < 0.01$ compared with baseline, * $P < 0.05$ compared with baseline.

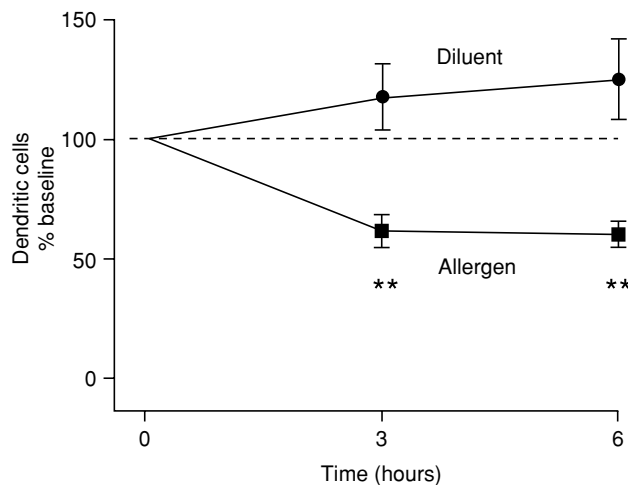
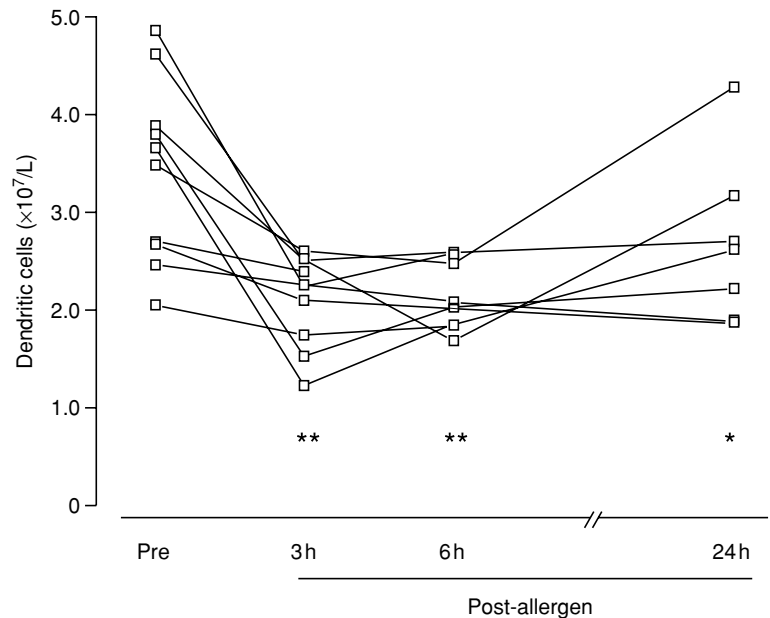


Fig. 3. Effects of diluent inhalation challenge on numbers of circulating dendritic cells. Diluent challenge with 0.9% saline was performed in six subjects. Data are expressed as percentage of cells numbers at baseline (i.e. prior to challenge). Both at 3 h and 6 h there was no significant change in numbers of circulating DC after diluent challenge.

disappeared from the circulation. Even at 24 h after allergen challenge, blood DC numbers remained below baseline values. In contrast, no such change in circulating DC was observed after diluent challenge.

We had anticipated that inhaled allergen might induce changes in circulating DC numbers, given that airway DC numbers are increased in stable allergic asthma [6, 7], and that a variety of inhaled stimuli increase DC numbers in the rat airway epithelium [4]. The surprising finding to emerge from the current study was the speed and magnitude of the change in circulating DC numbers.

While the exact fate of myeloid DC lost from the circulation after allergen challenge has not been determined in the current

study, it appears likely that these cells have margined within the bronchial and pulmonary vascular beds, and have begun to traffic into the airway, and possibly the alveoli. Indeed, Jahnsen et al. have recently shown increased numbers of myeloid DC in the asthmatic airway mucosa 4 to 5 hours after inhaled allergen [14]. A similar loss of circulating T cells and eosinophils after inhaled allergen challenge has been reported by others [15–18], and is confirmed in the current study (see Fig. 4). In contrast, we were unable to detect any appreciable change in blood $CD14^+$ monocyte counts after allergen challenge (Fig. 4), though this may simply mean that monocytes were entering and leaving the circulation at an equivalent rate.

Our findings do not of course preclude the possibility that some of the increase in airway DC numbers reported in stable asthma [6, 7] may also be attributable to the local differentiation of DC from precursor populations within the airway. While increased numbers of $CD34^+$ progenitor cells have been noted in asthmatic airways [19], the extent to which numbers of airway mucosal DC are regulated by local differentiation and by recruitment from the circulation are yet to be determined.

The mechanisms controlling the trafficking of DC into the lung are not fully elucidated, but are likely to involve the rapid up-regulation of adhesion molecules on both DC and endothelial cells, and the subsequent release of an array of chemoattractant molecules by various airway cells. DC are responsive to a variety of chemokines *in vitro*, including RANTES, eotaxin, MIP-1 α , MIP-3 α , MCP-1 and MCP-4 [4, 20, 21]. Many of these chemokines are present in inflamed asthmatic airways [22, 23], though which of these are most important in orchestrating DC recruitment after allergen challenge remains to be determined, once selective mediator antagonists become available.

It is acknowledged that certain limitations must be placed on the interpretation of our findings. First, while our experiments have focused on myeloid DC, the major subset of DC within the circulation, future studies will also need to examine the effects of allergen challenge on the rarer subset of IL-3 receptor-positive DC, also known as 'plasmacytoid' or 'lymphoid' DC [24]. It is intriguing that short-term allergen challenge

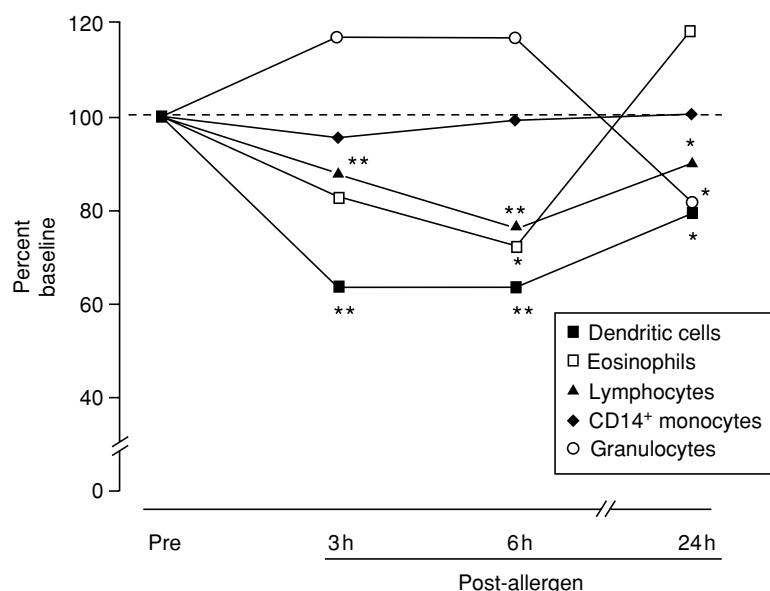


Fig. 4. Effect of allergen inhalation challenge on relative numbers of circulating leucocytes. Results are expressed as a percentage of baseline values prior to allergen challenge. * $P < 0.05$ vs. baseline, ** $P < 0.01$ vs. baseline.

to the lung does not alter the numbers of plasmacytoid DC in the bronchial mucosa [13], whereas repeated allergen challenge over 7 days is associated with recruitment of plasmacytoid DC into the nasal mucosa [13, 25]. It is possible that myeloid and plasmacytoid DC subsets differ in the speed with which they migrate to mucosal surfaces after allergen challenge. This issue can be most readily addressed in the future using repeated measurements of circulating DC, given the difficulties associated with performing repeated bronchoscopic biopsies in individuals with asthma.

Secondly, our experiments do not allow us to determine whether changes in blood DC after allergen inhalation challenge are most closely related to asthma or to atopy. Because an early fall in FEV₁ of at least 15% after allergen inhalation was a Key endpoint of the study, it was not practicable to administer an equivalent dose of allergen to both asthmatic and non-asthmatic subjects, nor was it possible to challenge subjects with an irrelevant allergen. Finally, the studies described herein do not allow us to examine possible links between changes in circulating DC and the development of the late asthmatic response. While a fall in blood DC numbers was observed in asthmatics exhibiting either early or dual responses to allergen inhalation, the number of early responders was small, and larger studies are warranted to address this issue.

Nonetheless, it remains highly likely that DC play an important role in the initiation and amplification of T cell-dependent immune responses within the airway. The potential importance of DC in asthma is underlined by fact that both allergen exposure (Fig. 2) and corticosteroid therapy [6] have such impressive effects on DC populations, and by the emerging functional differences between DC from allergic and non-allergic subjects [26]. Measurement of blood DC following allergen challenge is likely to become an important tool for examining the role of DC in asthma, particularly where repeated measurements are required over time, or in circumstances where bronchoscopy biopsy is not possible, given that DC can not be detected in induced sputum.

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