

Rapid recruitment of CD14⁺ monocytes in experimentally induced allergic rhinitis in human subjects

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Background: Activated T_H2 cells and eosinophils are hallmarks of the allergic inflammation seen in patients with allergic rhinitis (AR). However, which cells activate and attract T cells and eosinophils to the inflammatory lesion has not been determined.

Objective: We wanted to assess the role of mucosal mononuclear phagocytes, consisting of monocytes, macrophages, and dendritic cells, in the local allergic inflammatory reaction.

Methods: Patients with AR and nonatopic control subjects were challenged with pollen extract, and nasal symptoms were recorded. Mucosal biopsy specimens obtained at different time points before and after challenge were used for immunostaining *in situ* and flow cytometric cell sorting. Sorted mononuclear phagocytes were subjected to RNA extraction and gene expression profiling.

Results: In an *in vivo* model of AR, we found that CD14⁺ monocytes were recruited to the nasal mucosa within hours after local allergen challenge, whereas conventional dendritic cells accumulated after several days of continued provocation. Transcriptomic profiling of mucosal mononuclear phagocytes sorted after 1 week of continued allergen challenge showed an activated phenotype at least partially driven by IL-4 signaling, IL-13 signaling, or both. Importantly, gene expression of several T_H2-related chemokines was significantly upregulated by the mononuclear phagocyte population concomitant with an increased recruitment of CD4⁺ T cells and eosinophils.

Conclusion: Our findings suggest that the mononuclear phagocyte population is directly involved in the production of proinflammatory chemokines that attract other immune cells. Rapid recruitment of CD14⁺ monocytes to the challenged site

indicates that these proinflammatory mononuclear phagocytes have a central role in orchestrating local allergic inflammation. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: CD14⁺ monocytes, resident macrophages, dendritic cells, human airway allergy, nasal mucosa, T_H2-associated chemokines

Allergic diseases have become a public health concern of pandemic proportions. Allergic rhinitis (AR) alone affects 1 in 5 Europeans and is often a lifelong debilitating disorder with a major effect on quality of life. Moreover, AR and asthma are closely related diseases, and the majority of patients with allergic asthma have concomitant rhinitis.^{1,2}

In patients with AR, the inflammatory reaction is characterized by activated T_H2 cells, eosinophils, and mast cells. In addition, the human upper airway mucosa harbors a dense network of antigen-presenting cells (APCs) within and below the surface epithelium.³⁻⁵

These APCs can be assigned to the mononuclear phagocyte system (MPS). The MPS consists of monocytes, macrophages, and dendritic cells (DCs),⁶ which all exhibit multiple functions during an immune response. Cells of the MPS express MHC class II, thus being capable of presenting antigens to T cells. Furthermore, they produce cytokines and chemokines that activate and attract leukocytes.⁷ The APC network during homeostasis in the upper airway mucosa consists of spatially closely related macrophages and DCs.⁵ Mucosal DCs can be further divided into CD1c⁺ signal-regulatory protein α (SIRP α)⁺CD103⁺ and CD141⁺CD103⁺SIRP α ⁺ conventional dendritic cells (cDCs), CD103⁺SIRP α ⁺ DCs, and CD123⁺ plasmacytoid dendritic cells (pDCs).⁸ cDCs and pDCs originate from a common DC precursor in human subjects,⁹ whereas CD103⁺SIRP α ⁺ DCs are thought to be monocyte derived.⁸ Mucosal macrophages are stationary cells with important immunoregulatory functions. Although several studies in mice have shown that macrophages in various tissues are embryonically derived,¹⁰ mucosal macrophages appear to be constantly replenished by circulating monocytes,¹¹ and in human subjects it is suggested that incoming CD14⁺ monocytes can differentiate into 2 distinct populations: macrophages that maintain the expression of CD14 and CD103⁺SIRP α ⁺ DCs that become CD14⁺.

T_H2 cells are central to the inflammatory reaction in patients with AR. However, local activation of T_H2 cells depends on the presentation of allergenic peptides in the context of MHC class II. The MPS consists of professional APCs, and these cells are thus good candidates for activating T cells locally. Therefore detailed knowledge about the function of these upstream mediators of T-cell activation is important to understand the underlying immunopathology in patients with AR. Applying an *in vivo*

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Abbreviations used

APC:	Antigen-presenting cell
AR:	Allergic rhinitis
cDC:	Conventional dendritic cell
DC:	Dendritic cell
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
moDC:	Monocyte-derived dendritic cell
MPS:	Mononuclear phagocyte system
pDC:	Plasmacytoid dendritic cell
PNAd:	Vascular peripheral lymph node addressin
SIRPα:	Signal-regulatory protein α
SPT:	Skin prick test

human model of AR, we have shown that both CD1c⁺ cDCs and pDCs accumulate in the nasal mucosa after several days of repeated allergen challenge accompanied by typical allergic symptoms and eosinophilia.^{12,13} Recently, we have demonstrated that CD1c⁺ cDCs in the nasal mucosa express the receptor for thymic stromal lymphopoietin and that this proallergic cytokine increases their capacity to activate T_H2 cells.¹³ cDCs and pDCs have also been shown to play important roles in airway allergy in experimental mice; however, it was shown that monocyte-derived dendritic cells (moDCs) accumulate at high numbers during the effector phase of allergic inflammation and exhibit a strong capacity to recruit and activate T_H2 cells locally.¹⁴ In a rat model of allergic asthma, we have found that MHC class II⁺ cells (possibly monocyte derived) were rapidly recruited to the challenged tracheal mucosa and presented allergen to memory T cells.¹⁵ Examining severe asthma exacerbations in children, peripheral blood monocytes were expanded and showed signs of activation, including upregulation of CCR2, a receptor central for monocyte emigration.¹⁶ Together, these reports strongly suggested that recruitment of proinflammatory monocytes might be central players in allergic airway inflammation. Whether monocyte-derived cells play a similar role in patients with AR has not been investigated.

Functional characterization of specific cell types in human diseases is often restricted to *in situ* analysis of a few selected markers, which provides limited information. To obtain a more comprehensive study regarding the role of the MPS in patients with AR, we examined the composition of mononuclear phagocytes that accumulated in the nasal mucosa during experimentally induced AR and performed global transcriptomic profiling of the MPS isolated from the lesion.

METHODS**Ethics statement**

All participants in the study provided informed written consent. The study was approved by the Regional Committees for Medical and Health Research Ethics in Norway.

Patients

The size of nasal biopsy specimens obtained after topical anesthesia is limited, impeding the possibility to perform cell sorting and *in situ* immune staining in the same tissue specimen. We avoided taking more than 2 biopsy specimens from the same nostril to reduce the risk of incoercible epistaxis. Therefore 4 cohorts of adult patients (age >20 years) were necessary to complete this study.

In cohort I 8 patients with grass pollen allergy were recruited. The patients were allergen challenged according to the protocol shown in Fig E1, A, in this article's Online Repository at www.jacionline.org. In cohort II 9 patients with grass pollen allergy and 5 healthy nonatopic control subjects were included and challenged (see Fig E1, B). Cohort III comprised 6 patients with birch pollen allergy whose tissue specimens were processed for cell sorting before and after the allergen provocation protocol (see Fig E1, C). In cohort IV 8 patients with birch pollen allergy were recruited and challenged (see Fig E1, D). Nasal biopsy specimens obtained for *in situ* staining were either snap-frozen bedside (cohort I and II) or formalin fixed and paraffin embedded (cohort IV).

All patients were challenged according to the same procedure, as described below. Applying the same *in vivo* provocation model, we previously showed that both pollen allergens induce similar symptoms and blood and tissue eosinophilia in challenged allergic patients.¹² All patients fulfilled the Allergic Rhinitis and its Impact on Asthma criteria¹⁷ for persistent moderate-to-severe AR and had been symptomatic for at least the last 3 pollen seasons.

In cohorts I and II all allergic patients had positive skin prick test (SPT) responses to grass pollen. Four subjects in cohort I and 5 subjects in cohort II also had positive SPT responses to birch, house dust mites, and/or cat, dog, or horse dander. All control subjects (cohort II) had negative responses to all tested aeroallergens. Patients in cohorts III and IV had positive SPT responses to birch pollen. Four subjects in cohort III and 5 subjects in cohort IV were also sensitized to grass, mugwort, ragweed, house dust mites, and/or cat, dog, or horse dander.

Any other types of nasal disorder were excluded based on both the patient's history and clinical examination, including nasal endoscopy. Nasal provocation was performed out of the pollen season. No patients experienced nasal allergic symptoms in the months before provocation or received any medication during the challenge period. Control subjects did not have any allergic or nasal disease, and the patients and control subjects were otherwise healthy. None of the participants were smokers.

Nasal allergen challenge

The nasal challenge procedure has been described in detail elsewhere.¹² A solution of pollen extract was delivered once a day into one nostril with a hand-driven pump spray, producing a defined volume of 50 µL (100,000 SQ/mL, either of Aquagen grass or Aquagen birch; ALK-Abelló, Hørsholm, Denmark).

Nasal symptoms

Patients were instructed to score each nasal symptom (sneezing, rhinorrhea, and nasal blockage). For patients in cohorts I and II, this was recorded daily on a 4-point scale (range, 0-3). The subjects in cohorts III and IV used a 10-cm visual analog scale ranging from "not bothered at all" to "extremely bothered."

Nasal biopsy specimens

Before nasal challenge (day 0), a mucosal biopsy specimen was obtained after achievement of topical anesthesia (tetracaine/adrenaline) from the lower turbinate of one nostril. Thereafter, allergen challenge was performed on the other nasal cavity according to the corresponding protocol (see Fig E1). The following nasal biopsy specimens were always taken after achievement of topical anesthesia from the lower turbinate of the challenged nostril. For cohorts I, III, and IV, the last biopsy specimen was obtained 24 hours after the final allergen dose. No biopsy-related complications occurred.

Tissue preparation for cell sorting and gene expression profiling

The biopsy specimens were immediately digested for 60 minutes at 37°C in Dulbecco modified Eagle medium (DMEM; Lonza, Basel, Switzerland) containing 2 mg/mL Liberase TL (Roche Diagnostics, Basel, Switzerland) to release single cells. Before flow cytometric sorting, cells were stained with the antibodies listed in Table E1 in this article's Online Repository at www.jacionline.org.

[jacionline.org](http://www.jacionline.org). TOPRO-1 Iodide (515/531; Invitrogen, Life Technologies, Grand Island, NY) was used for live/dead cell discrimination. Stained cells were sorted with a FACSAria (BD Biosciences, San Jose, Calif) and immediately lysed in TRIzol (Life Technologies). Cells were sorted as TOPRO-1⁻CD19⁻CD3⁻CD45⁺HLA-DR⁺ cells (see Fig E2 in this article's Online Repository at www.jacionline.org). The purity of sorted populations was routinely greater than 94%.

Gene expression profiling

Total RNA was extracted from TOPRO-1⁻CD19⁻CD3⁻CD45⁺HLA-DR⁺ cells with TRIzol, followed by RNeasy. The RNA samples were amplified and labelled with the NuGEN Ovation Pico WTA system with the WT-Ovation Exon Module and the Encore Biotin Module at the Ramaciotti Centre (University of New South Wales, Kensington, Australia). CD45⁺HLA-DR⁺ cells were hybridized to Human Gene 1.1 ST arrays (Affymetrix, Santa Clara, Calif), and the raw microarray data were available at the Gene Expression Omnibus (GSE49782). The quality of the microarray data was assessed in Expression Console (Affymetrix) and R software. A multivariate outlier detection procedure (Mahalanobis Distance Quality Control)¹⁸ was used to identify low-quality samples based on a series of quality metrics (positive vs negative area under the curve, relative log expression, and normalized unscaled SEs). Low-quality samples were removed from analysis.

Microarray data analysis

The raw microarray data were preprocessed in R software by using the single-channel array normalization algorithm. A custom gene definition file (hugene10sthsentrezg, hugene11sthsentrezg, version 17) was used to map probes to genes based on current genome knowledge. The universal probability of expression codes algorithm (an extension of the single-channel array normalization algorithm)¹⁹ was used to identify probes sets that were expressed at greater than background and to filter data (threshold probability ≥ 0.25 in $\geq 25\%$ of the samples). Differentially expressed genes were identified by using Bayesian/moderated *t* statistics (LIMMA).

Bioinformatics

Biological functions enriched in the data were identified by using the database for annotation, visualization, and integrated discovery. The data were also interrogated with gene sets from the molecular signatures database. Ingenuity Systems (Redwood City, Calif) software was used for pathways analysis and upstream regulator analysis. A molecular interaction network was reconstructed from the differentially expressed genes by using prior knowledge from the Ingenuity Knowledge Base.

Tissue preparation for multicolor immunofluorescence staining

The mucosal samples in cohorts I and II were embedded in OCT medium and snap-frozen in liquid nitrogen. The biopsy specimens from cohort IV were fixed in formalin overnight and subsequently embedded in paraffin. Multicolor immunostaining was performed, as described elsewhere.⁵ The mAbs used are listed in Tables E2 and E3 in this article's Online Repository at www.jacionline.org. To determine the fraction of CD4⁺ T cells that expressed GATA3, we costained serially cut sections with anti-CD3 and anti-CD4 or anti-CD3 and anti-GATA3, respectively. Because GATA3 is not expressed by CD8⁺ T cells, we estimated the percentage of GATA3-expressing CD4⁺ T cells by dividing the density of CD4⁺ T cells by the density of GATA3⁺ T cells on neighboring sections. Sections were stained with BSA-fluorescein isothiocyanate for 1 hour to enumerate eosinophils. BSA-fluorescein isothiocyanate specificity for eosinophils was reassured by costaining sections of nasal polyps with an mAb directed to eosinophil major basic protein (see Table E2). A complete overlap between the 2 markers was observed (not shown). Concentration-matched irrelevant isotype antibodies were used as negative controls.

Immunofluorescence microscopy

The slides were examined by 2 investigators (I.E.-G. and G.R.M.) at $\times 40$ magnification with a fluorescence microscope. Cells were enumerated by superimposing a grid (250 $\times$ 250 μ m) parallel to the basement membrane of the surface epithelium to determine cell densities in the epithelium and mucosa.

Flow cytometric analysis of PBMCs and nasal mucosa-derived cells

PBMCs were obtained from healthy blood donors ($n = 3$). Normal mucosal biopsy specimens were obtained from the inferior turbinate during septal deviation surgery in otherwise healthy donors ($n = 3$; age range, 20-52 years). Single cells for flow cytometric analysis were prepared, as previously described,¹³ and stained for 30 minutes at 4°C with the antibodies listed in Table E1. Irrelevant isotype- and concentration-matched antibodies were used for controls, and TOPRO-1 (Thermo Fisher Scientific, Waltham, Mass) was used for live/dead discrimination.

Allergen treatment of moDCs

To generate dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)⁺ moDCs, CD14⁺ monocytes were purified from PBMCs of healthy blood donors with the Dynabeads Untouched Human Monocytes Kit (Thermo Fisher Scientific). Cells were cultured for 48 hours in the presence of 800 U of recombinant human GM-CSF and 1000 U of recombinant human IL-4 (both from R&D Systems, Minneapolis, Minn) in RPMI-1640 medium (Lonza) supplemented with 2 μ mol/L glutamine and 10% FBS (Gibco, Carlsbad, Calif). The DCs were then treated with 10 μ g/mL allergen solution (Aquagen timothy or Aquagen birch, ALK-Abelló) in culture medium for 120 minutes, stained for 30 minutes at 4°C with anti-DC-SIGN (see Table E1), and subsequently analyzed by means of flow cytometry (LSR Fortessa, BD Biosciences).

Statistics

GraphPad Prism software (version 5.0; GraphPad Software, La Jolla, Calif) was used for statistical analysis. The distribution of the samples was assessed by using the Kolmogorov-Smirnov test. Comparisons between normally distributed samples were made by using either 1-way ANOVA (followed by the Newman-Keuls test) or the Student *t* test for paired samples. For samples not normally distributed, either the Kruskal-Wallis test (followed by the Dunn test) or the Wilcoxon test was used. A *P* value of less than .05 was considered significant.

RESULTS

Rapid recruitment of CD14⁺ monocytes at the site of allergen challenge in patients with AR

We previously showed that the density of pDCs and CD1c⁺ cDCs, but not CD141⁺ cDCs, increases in the nasal mucosa of patients with AR after 7 days of allergen challenge.^{12,13} However, whether recruitment of CD14⁺ monocytes is a part of the allergic inflammatory reaction has not been investigated. To this end, we performed a 7-day challenge protocol and obtained mucosal biopsy specimens at baseline and at days 3 and 8 (cohort I). All patients experienced typical symptoms (see Fig E3, A, in this article's Online Repository at www.jacionline.org), and increased numbers of tissue eosinophils were observed at both time points (see Fig E4, A, in this article's Online Repository at www.jacionline.org), demonstrating that our model resulted in a typical allergic reaction. Costaining tissue sections with mAbs to HLA-DR and CD14 revealed a significant ($P < .01$) 8-fold increase in the number of CD14⁺HLA-DR⁺ cells at day 3, which was maintained after 1 week of continued provocation (Fig 1, A). In

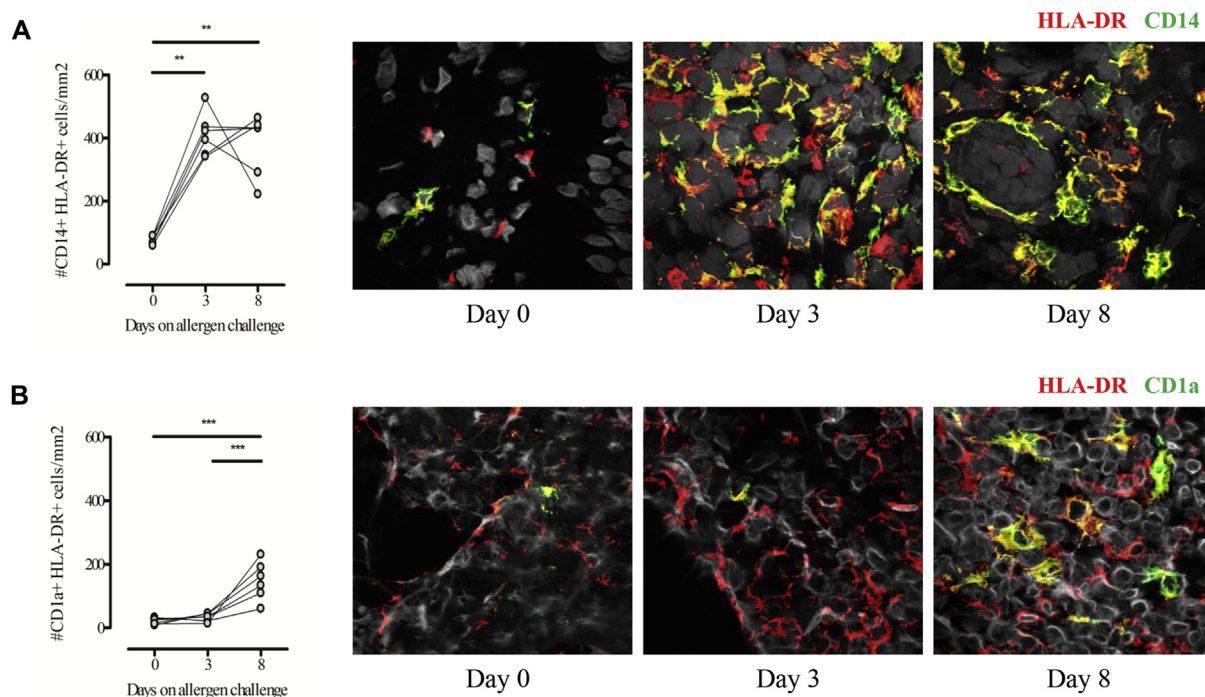


FIG 1. CD14⁺HLA-DR⁺ cells accumulate in challenged nasal mucosa from allergic patients earlier than CD1a⁺HLA-DR⁺ cells (cohort I). **A**, Density of CD14⁺HLA-DR⁺ cells at baseline and after 3 and 8 days of challenge (left) and immunofluorescence staining of HLA-DR and CD14 (right). **B**, Density of CD1a⁺HLA-DR⁺ cells at baseline and after 3 and 8 days of allergen challenge (left) and immunofluorescence staining of HLA-DR and CD1a (right). ** $P < .01$ and *** $P < .001$.

comparison, CD1a⁺HLA-DR⁺ DC numbers also showed a significant increase at day 8 (Fig 1, B), but no change was found at day 3 compared with baseline values.

The massive accumulation of CD14⁺HLA-DR⁺ cells in response to allergen challenge strongly suggested an increased recruitment of CD14⁺ monocytes from the circulation, preceding that of cDCs. CD14⁺HLA-DR⁺ cells were negative for the proliferation marker Ki67 at all time points (data not shown), further underscoring this notion.

The different kinetics between CD14⁺ monocytes and cDCs prompted us to examine the density of CD14⁺HLA-DR⁺ cells at earlier time points. We performed a new challenge study (cohort II) in which nasal biopsy specimens were obtained at 4 and 12 hours after a single challenge dose. Because resident macrophages in the nasal mucosa also express CD14 (Melum et al, unpublished data), we took advantage of the observation that most macrophages express DC-SIGN/CD209, both in the intestinal²⁰ and nasal mucosa (see Fig E5, B), whereas circulating CD14⁺ monocytes express low levels of this protein (see Fig E5, A).²¹ At baseline, most CD14⁺HLA-DR⁺ cells coexpressed DC-SIGN, and there were no differences in the numbers of either CD14⁺DC-SIGN⁺ or CD14⁺DC-SIGN⁻ cells comparing values at baseline with those 4 hours after challenge (Fig 2, A). However, 12 hours after challenge, the density of CD14⁺DC-SIGN⁻ cells was significantly increased, whereas the density of CD14⁺DC-SIGN⁺ cells was unchanged (Fig 2, B). In contrast, no change in CD14⁺ cell densities was observed in the nonatopic control group (Fig 2, C), strongly suggesting that the increase in CD14⁺DC-SIGN⁻ cell numbers was dependent on the allergic inflammatory reaction and not related to the biopsy procedure. It has been reported that allergen binding could alter surface

expression of DC-SIGN on CD14⁺ cells.²² To investigate this, blood monocytes were cultured with GM-CSF and IL-4 to induce DC-SIGN expression and incubated with birch and grass extracts. However, no reduction of surface staining for DC-SIGN was observed (see Fig E6 in this article's Online Repository at www.jacionline.org). Similar to cohort I, the density of eosinophils in patients with AR (cohort II) paralleled that of CD14⁺DC-SIGN⁻ cells, increasing significantly at 12 hours after challenge (see Fig E4, C), whereas no change in eosinophil numbers was observed in the control group (see Fig E4, C). Recently, it was shown that also pDC numbers rapidly increase in the skin and nasal mucosa after allergen challenge in atopic patients.²³ However, costaining for CD123 and CD45RA, which identifies pDCs,¹² we found the density of pDCs to be low and unchanged at both 4 and 12 hours compared with baseline values (see Fig E7 in this article's Online Repository at www.jacionline.org). Together, these findings strongly suggest that peripheral blood CD14⁺ monocytes were recruited within hours to the challenged nasal mucosa in patients with AR, whereas the density of tissue macrophages, identified as CD14⁺DC-SIGN⁺ cells, remained unaltered.

Transcriptomic profiling shows signs of activation of the MPS in the nasal mucosa after allergen challenge

Despite the described accumulation of cells of the MPS during the allergic reaction, their functional role is largely undetermined. To address this issue, we performed an allergen challenge study (cohort III) in which we sorted the MPS from the allergen-challenged nasal mucosa for global transcriptomic analysis.

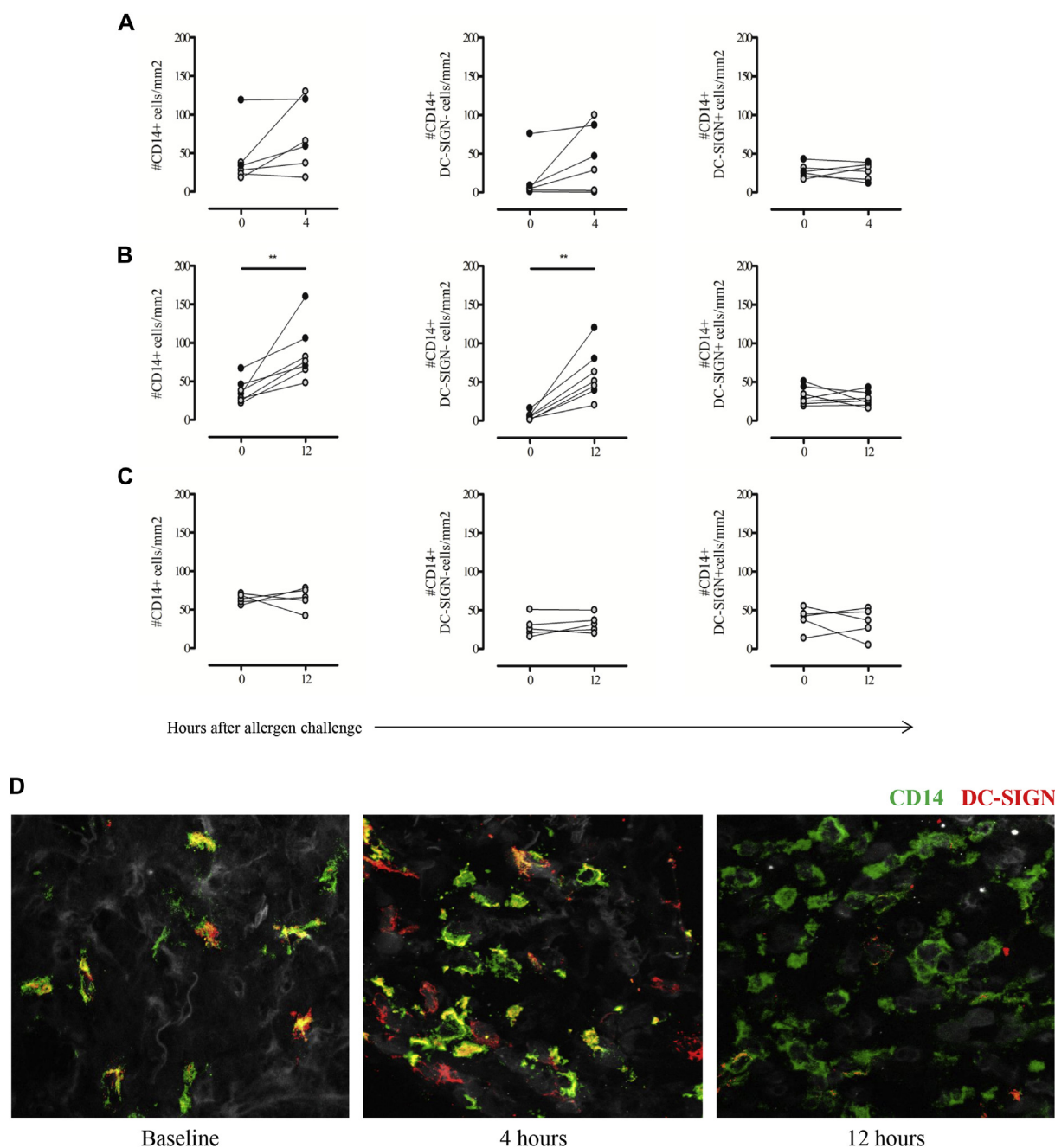


FIG 2. Monocyte numbers increase in nasal mucosa of allergic patients 12 hours after challenge, whereas numbers of tissue macrophages are unchanged (cohort II). **A-C.** Density of CD14⁺ cells, CD14⁺DC-SIGN⁺ monocytes, and CD14⁺DC-SIGN⁺ macrophages at baseline and 4 hours after challenge in allergic patients (Fig 2, A), 12 hours after challenge in allergic patients (Fig 2, B), and 12 hours after challenge in control subjects (Fig 2, C). **D.** Immunofluorescence staining of CD14 and DC-SIGN. In Fig 2, A and B, gray symbols represent patients analyzed at all 3 time points (baseline and 4 and 12 hours), whereas black symbols represent patients examined only at 2 time points (baseline and either 4 or 12 hours). ***P* < .01.

Because the sample size of nasal mucosal biopsy specimens is limited and the frequency of tissue APCs is low, sorting cell subsets of the MPS separately was not feasible. Therefore we decided to sort all CD45⁺HLA-DR⁺ cells after an 8-day provocation protocol. At this time point, CD1c⁺ cDCs, pDCs, and CD14⁺HLA-DR⁺ monocyte-derived cells were increased in number.^{12,13} Expression profiling of sorted TOPRO-1[−]CD19[−]CD3[−]CD45⁺HLA-DR⁺ cells identified a total of 63

differentially regulated genes between baseline and day 8: 51 were upregulated, and 12 were downregulated (see Fig E8 in this article's Online Repository at www.jacionline.org). A number of the differentially expressed genes are regulated by IL-4 and IL-13 signaling (Fig 3 and see Table E4 in this article's Online Repository at www.jacionline.org), including several chemokines (CCL13, CCL17, CCL18, and CCL24) and components of the CD1 antigen presentation pathway, such as CD1a and

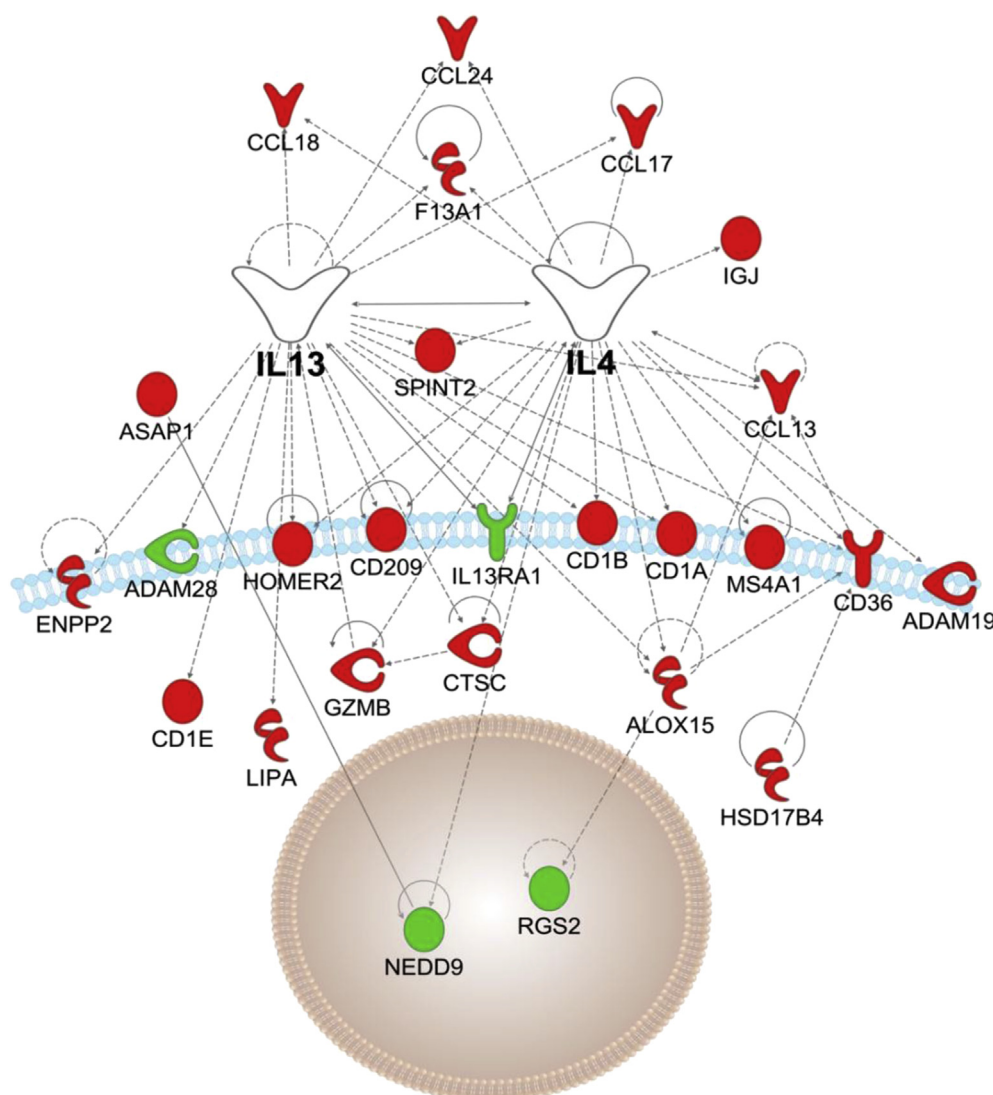


FIG 3. Molecular interaction network in TOPRO-1⁺CD19⁺CD3⁺CD45⁺HLA-DR⁺ cells after allergen (cohort III). Mechanistic data from previous studies was used to build a molecular interaction network. For clarity, the figure illustrates genes downstream of IL-4 and IL-13 signaling. Genes are shown according to their cellular compartmental location (nucleus, cytoplasm, plasma membrane, and extracellular space). Genes colored *red* were upregulated, and genes colored *green* were downregulated.

CD1b. Increased mRNA expression of CD1a was in agreement with results of our *in situ* staining, showing increased density of CD1a⁺ cDCs after 1 week of challenge (Fig 1, B). To further validate the microarray data, we also examined the *in situ* expression of CD1b on HLA-DR⁺ cells. Immunofluorescence staining showed that the density of CD1b⁺HLA-DR⁺ cells was significantly increased after 7 days of challenge but not at day 3 (Fig 4), which was similar to the kinetics for CD1a⁺ cDCs. Together, these results demonstrate that the MPS in the allergen-challenged nasal mucosa displays an activated phenotype that is partially driven by IL-4 signaling, IL-13 signaling, or both.

CD4⁺ T cells are recruited to the site of allergen challenge in patients with AR

Increased expression of T cell-attracting chemokines led us to examine whether T cells accumulated in our model of allergen

challenge. We found a robust increase in CD4⁺ T-cell numbers at both days 3 and 8 after challenge (cohort I; Fig 5, A), whereas no increase in CD8⁺ T-cell numbers was observed at any time point (Fig 5, C). To determine whether this accumulation was due to increased recruitment, we examined the *in situ* expression of L-selectin/CD62 ligand on CD4⁺ T cells. L-selectin is a ligand for vascular peripheral lymph node addressins (PNAds), and their interaction is important for leukocyte recruitment to organized lymphoid tissues and in various inflammatory settings.²⁴ Importantly, we have shown that PNAds are induced on nasal mucosal venules in patients with AR.¹² Moreover, L-selectin is rapidly downregulated when T cells are activated in tissues, suggesting that L-selectin marks recently recruited T cells.²⁵ We found that only 10% of mucosal CD4⁺ T cells expressed L-selectin at baseline. However, after 3 and 7 days of allergen challenge, the fraction of L-selectin-positive CD4⁺ T cells increased significantly ($P \leq .01$ and $P < .001$, respectively) and paralleled the total number

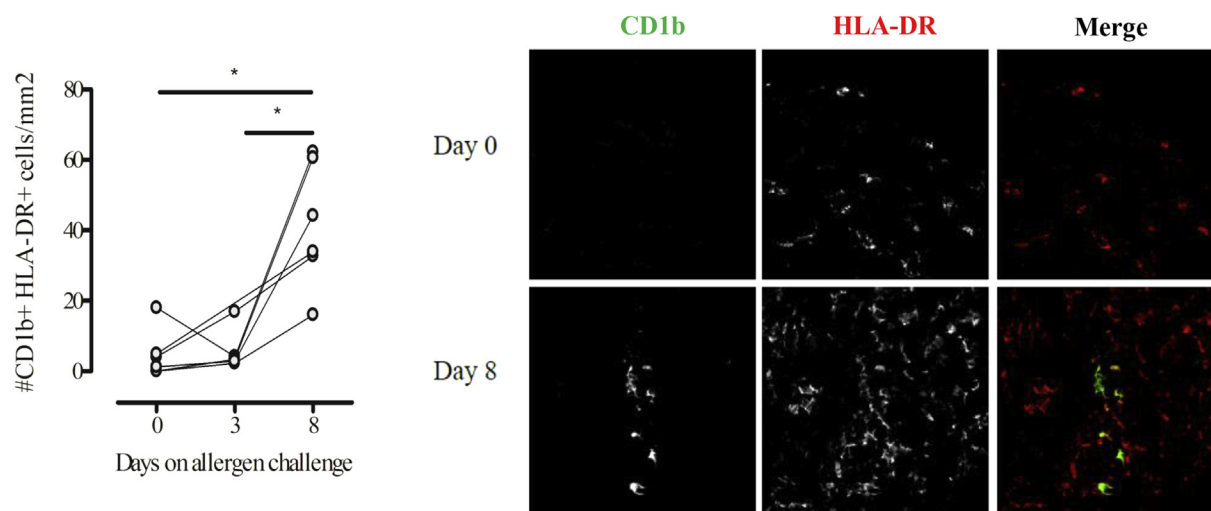


FIG 4. CD1b⁺HLA-DR⁺ cells accumulate in the nasal mucosa from allergic patients after 8 days of challenge (cohort I). *Left*, Density of CD1b⁺HLA-DR⁺ cells at baseline and after 3 and 8 days of allergen challenge. *Right*, Immunofluorescence staining of CD1b and HLA-DR. **P* < .05.

of CD4⁺ T cells, indicating that a large proportion of CD4⁺ T cells in the challenged mucosa were recent emigrants (Fig 5, B).

The finding of an IL-4/IL-13 signature in the MPS after 1 week of allergen challenge prompted us to examine whether we could detect increased numbers of T_H2 cells. The transcription factor GATA3 is a good marker for T_H2 cells; however, staining for GATA3 on frozen tissue sections (cohorts I and II) produced suboptimal results. Therefore we examined a new cohort (cohort IV) consisting of 8 patients with AR from whom formalin-fixed mucosal biopsy specimens were obtained before and after 2 consecutive days of challenge with birch pollen. All the patients experienced typical allergic symptoms (see Fig E3, C), and a significant increase in numbers of tissue eosinophils was observed (see Fig E4, B). Importantly, the proportion of GATA3⁺ cells among all CD4⁺ T cells was significantly increased after provocation compared with baseline (Fig 6), suggesting a preferential accumulation of T_H2 cells. The density of CD14⁺ monocytes was also significantly increased (*P* < .001), whereas the number of resident macrophages did not change after challenge, further corroborating our results in cohorts I and II.

Together, these observations strongly suggest that circulating CD4⁺ T cells are recruited in patients with AR and that accumulating T_H2 cells might be involved in regulating the function of the MPS.

DISCUSSION

This study shows that CD14⁺ monocytes are rapidly recruited (within hours) to the allergen-challenged mucosa in patients with AR, whereas CD1c⁺ cDC numbers increase after several days of continuous exposure. Transcriptional analysis of mononuclear phagocytes sorted from the challenged nasal mucosa showed hallmarks of activation and maturation that were partially regulated by IL-4/IL-13 signaling. The IL-4/IL-13-dependent signature included multiple chemokines involved in T_H2 responses.

Work from our group has shown that HLA-DR⁺ leukocytes in the normal nasal mucosa consist mainly of macrophages and

CD1c⁺ cDCs.⁵ We and others have shown that the total number of HLA-DR⁺ leukocytes increases after allergen provocation in experimental models of AR.^{13,26} Here we show that most of the accumulating HLA-DR⁺ leukocytes coexpress CD14, and the rapid increase after a single allergen exposure suggests that these CD14⁺ monocytes are being recruited from the circulation. This finding is consistent with animal models of asthma showing a rapid increase in numbers of MHC class II⁺ DCs in the airways after bronchial allergen challenge.^{14,15} Interestingly, it was shown that this increase was mainly due to an accumulation of moDCs that, unlike CD11b⁺ cDCs, display limited migratory capacity but were very efficient in driving inflammation locally.¹⁴ Therefore it is reasonable to speculate that the CD14⁺ mononuclear phagocytes that accumulated in our AR model play a similar role.

CD14⁺ monocytes in the circulation express a variety of chemokine receptors, of which CCR2 and CX3CR1 are thought to be crucial for their emigration to tissues.²⁷ Studies of AR have shown that ligands for these receptors, CCL13 and CX3CL1, are both secreted from the respiratory mucosa in response to a single allergen dose.^{28,29} These findings suggest that these chemokine receptor–ligand pairs could be involved in the rapid recruitment of monocytes observed in our study.

During homeostasis, incoming monocytes to the intestinal mucosa differentiate into anti-inflammatory macrophages.¹¹ However, this differentiation program is arrested during inflammation, where recruited monocytes differentiate into proinflammatory macrophages¹¹ or inflammatory DCs.³⁰ Together, these findings suggest that the function of monocytes recruited to tissue sites is context dependent; under homeostatic conditions, they become anti-inflammatory macrophages, whereas in an inflammatory microenvironment they differentiate into inflammatory mononuclear phagocytes.

A rat model of allergic asthma with prolonged aeroallergen exposure showed that the inflammatory reaction was antagonized by accumulating regulatory T cells.³¹ Although we also have observed increased numbers of forkhead box protein 3–positive regulatory T cells after 7 days of continuous challenge in our AR model,³² we did not detect a reduction in tissue eosinophil

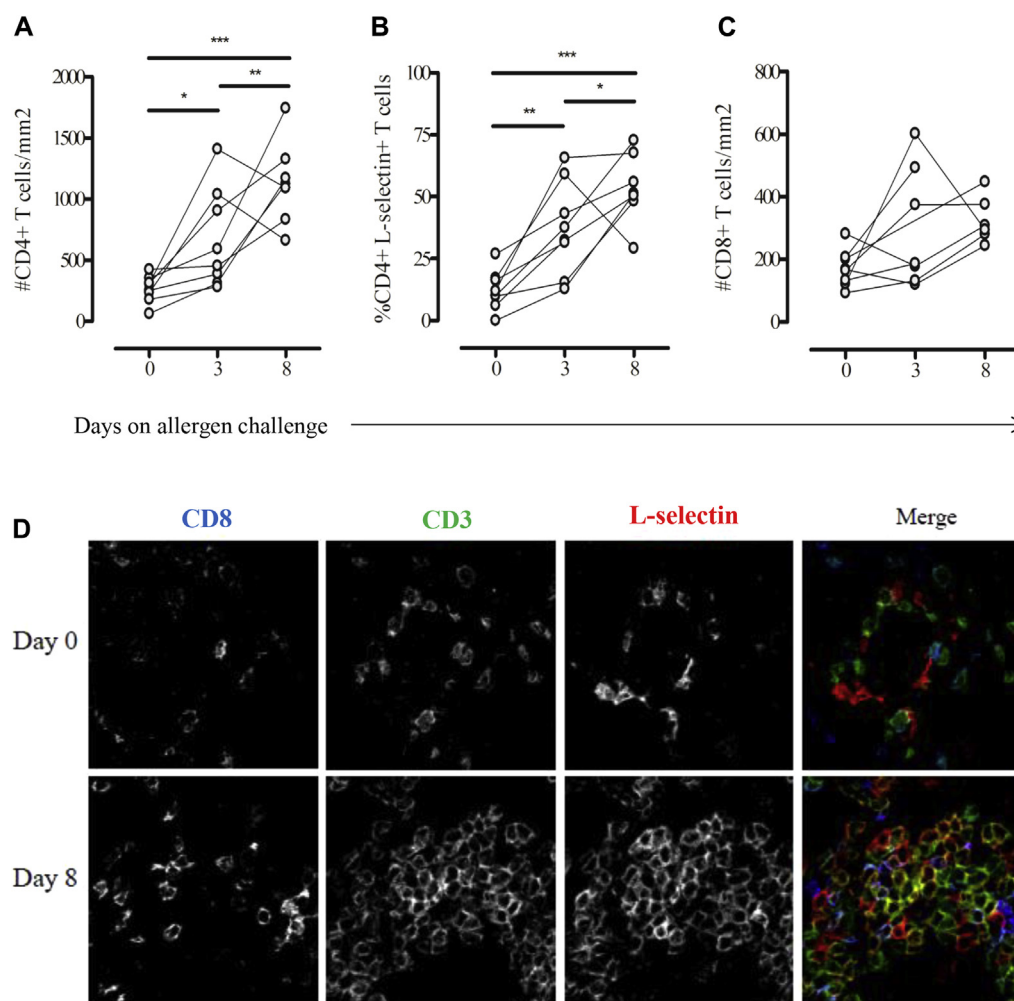


FIG 5. Numbers of newly recruited CD4⁺ T-cells increase in the nasal mucosa of allergic patients after 3 days of challenge (cohort I). **A-C**, Density of CD4⁺ T cells (Fig 5, A), proportion of CD4⁺ T cells expressing L-selectin (Fig 5, B), and density of CD8⁺ T cells (Fig 5, C) at baseline and after 3 and 8 days of challenge. **D**, Fluorescence immune staining of CD8, CD3, and L-selectin. **P* < .05, ***P* < .01, and ****P* < .001.

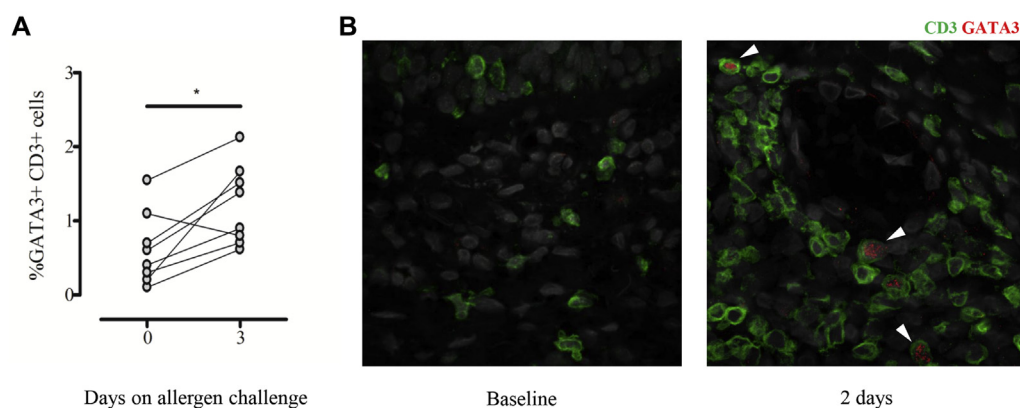


FIG 6. The proportion of TH2 cells is increased in the allergen-challenged nasal mucosa (cohort IV). **A**, Proportion of GATA3⁺ cells among CD4⁺ T cells at baseline and after 2 consecutive days of allergen challenge. **B**, Immunofluorescence staining of CD3 and GATA3. **P* < .05.

counts or symptoms at this time point³² (and in this study). However, because of differences between species, tissues, and allergens, these models are difficult to compare.

We found 63 genes within the MPS that were differentially expressed after 1 week of allergen challenge compared with baseline. Almost half of these genes are regulated by either IL-4,

IL-13, or both,^{33,34} and within this group, 4 chemokine genes were upregulated. Importantly, all these chemokines (CCL13, CCL17, CCL18, and CCL24) are ligands for chemokine receptors (CCR3, CCR4, and CCR8) that are typically expressed on differentiated T_H2 cells.³⁵

A role for these T_H2 -associated chemokines was further supported by an increased density of $CD4^+$ T cells after challenge, in which an increasing fraction expressed the T_H2 -associated transcription factor GATA3. Importantly, a large fraction of these $CD4^+$ T cells expressed L-selectin. L-selectin-mediated migration of T cells has been observed under various inflammatory situations,³⁶ and postcapillary venules have been reported to upregulate PNADs in allergen-challenged nasal mucosa, suggesting that L-selectin/PNAD interactions might play a role in recruitment of T cells to this site.¹² Furthermore, L-selectin is downregulated on T cells after activation in tissues, enabling the use of L-selectin as a marker of recently recruited T cells. Therefore our observations suggest that a large fraction of accumulating $CD4^+$ T cells, which included an increased number of T_H2 cells, had been recently recruited.

We have recently shown that more than 50% of $CD4^+$ T cells in the normal nasal mucosa express CCR3 compared with less than 0.5% in the bloodstream, indicating that CCR3 is also important for the homing of $CD4^+$ T cells under homeostatic conditions.³⁷ Together, these observations indicate that in the upper airways CCR3 on T cells might act both as a tissue-tropic molecule under homeostatic conditions and as a recruiter of T cells during allergic inflammation.

In this study we were not able to determine which cell subsets within the MPS were responsible for increased expression of the T_H2 -associated chemokine genes. Monocytes, DCs, and macrophages have been shown to express T_H2 chemokines under T_H2 -polarizing conditions.³⁸ Interestingly, moDCs in mouse models of allergic asthma produced several proinflammatory chemokines, such as CCL24.¹⁴ Thus it is tempting to speculate that the accumulating $CD14^+$ mononuclear phagocytes observed in our AR model play a role in orchestrating the allergic reaction locally.

The gene expression signature of $CD45^+HLA-DR^+$ cells was also characterized by upregulation of genes belonging to the lipid antigen presentation pathway. CD1a and CD1b are members of the CD1 family of transmembrane glycoproteins and are structurally related to the components of the MHC.³⁹ We found increased expression of CD1a and CD1b both at the mRNA and protein levels. Expression of these proteins is associated with DC maturation⁴⁰ and implies that these cells respond to allergen challenge by increasing their capacity to present allergens to allergen-specific T cells. In line with this, we have previously shown that expression of CD1a in the nasal mucosa is restricted to a subset of $CD1c^+$ cDCs that significantly increases after allergen provocation.¹³

In conclusion, the gene expression profile of the MPS concomitant with increased recruitment of $CD14^+$ monocytes and $CD1c^+$ cDCs implies that these APCs are key players in allergic reactions both based on their capacity to present antigenic peptides to allergen-specific T cells and their ability to recruit and activate immune cells in the lesion (eg, T_H2 cells and eosinophils). This notion is in accordance with recent mouse models demonstrating that recruited moDCs and $CD11b^+$ cDCs are both important drivers of allergic asthma. Further research exploring the therapeutic potential of antagonizing the activation of the MPS during upper airway allergy might provide treatment targets.

We thank C. Westin and R. Blumberg for providing DC-SIGN and CD1b antibodies, respectively, and Kjersti Thorvaldsen Hagen and Aaste Aursjø for their excellent technical assistance.

Clinical implications: Our study supports the rationale of targeting monocyte-derived APCs to ameliorate allergic inflammation in patients with AR.

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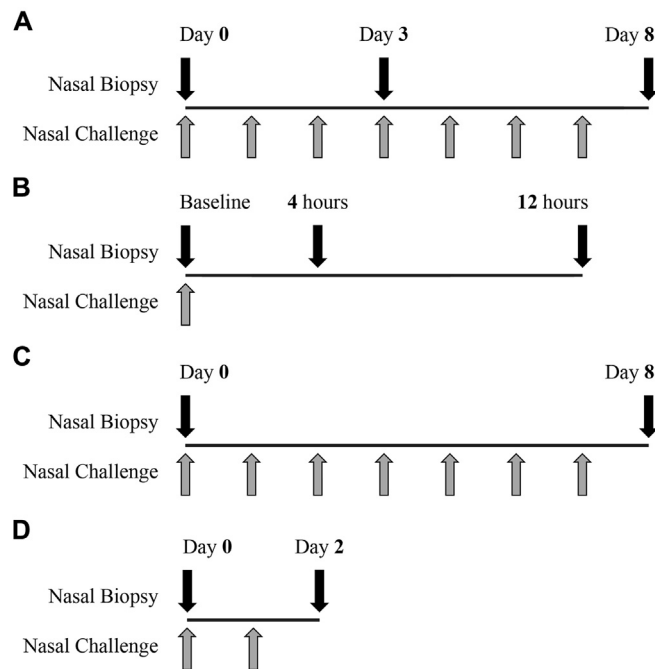


FIG E1. Nasal provocation protocols of the 4 cohorts included in the study. **A**, Cohort I; **B**, cohort II; **C**, cohort III; and **D**, cohort IV.

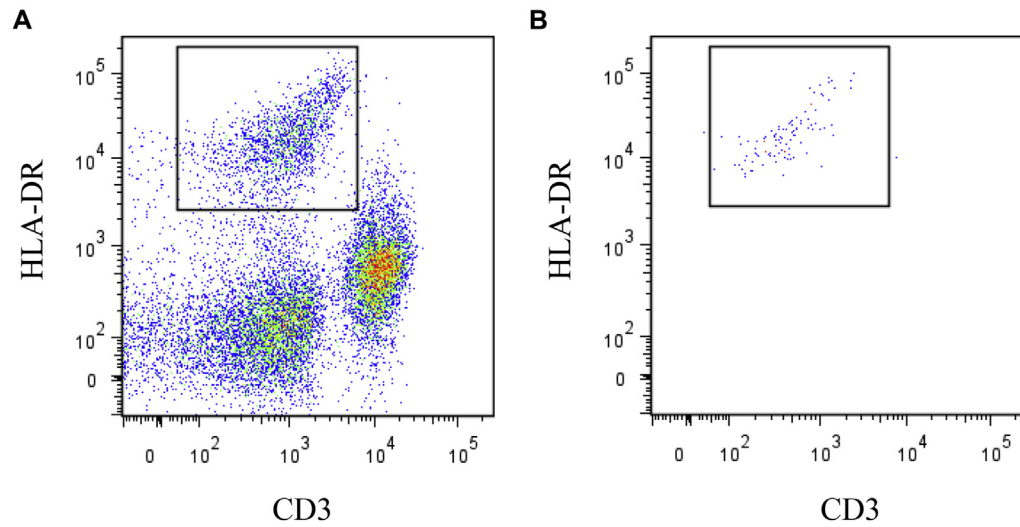


FIG E2. Gating procedure and purity analysis of sorted cells (cohort III). Within the cells released by means of Liberase TL digestion, a gate was first set to include cells expressing CD45 but exclude CD19⁺ cells and the dead cell discriminator TOPRO-1. Within this population, APCs were gated as HLA-DR⁺CD3⁻ cells (A). Analysis of sorted APCs consistently revealed purities of greater than 94% (B).

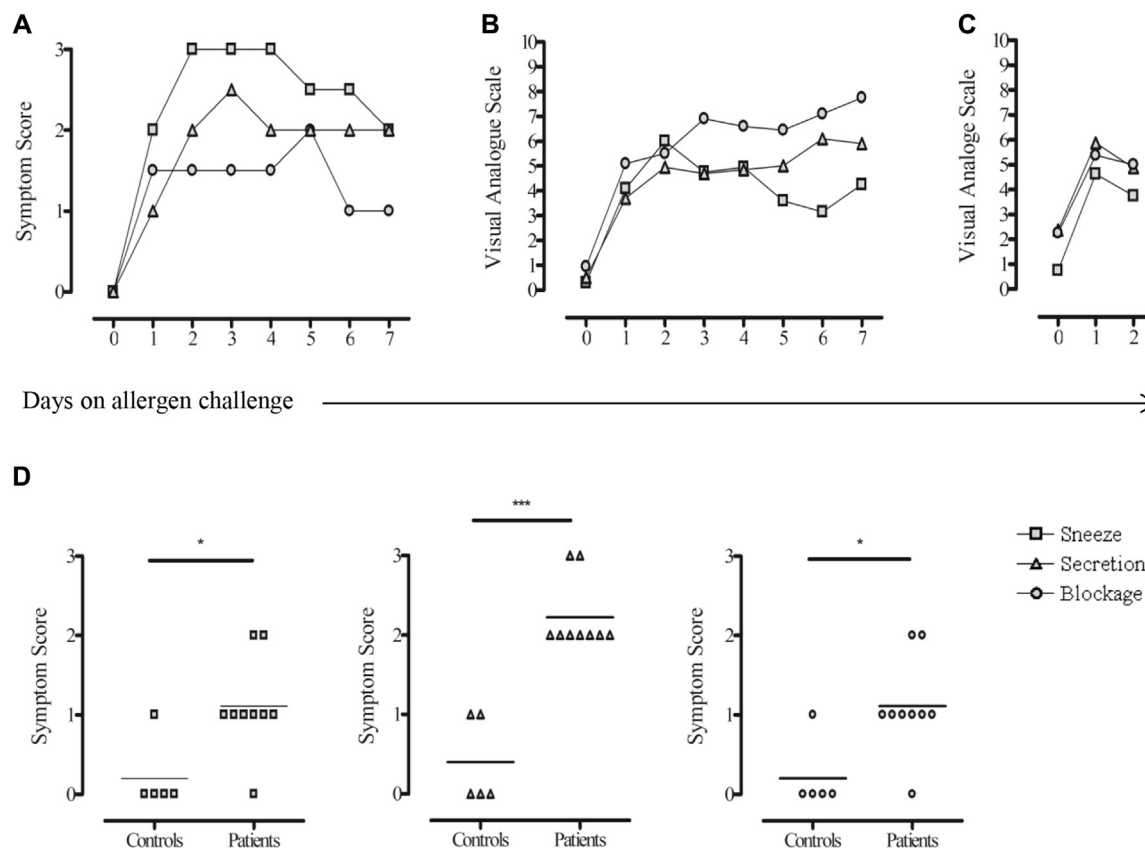


FIG E3. All nasal provocation protocols induced typical allergic symptoms in challenged patients. **A**, Symptom score from patients in cohort I. **B**, Visual analog scale from patients in cohort III. **C**, Visual analog scale from patients in cohort IV. The median of patient symptoms for each day is represented for the 3 examined parameters. **D**, Symptom score for each of the 3 examined parameters 15 minutes after allergen challenge in subjects from cohort II. In cohort II symptom scores for all the subjects at baseline was 0 for the 3 examined parameters. * $P < .05$ and *** $P < .001$.

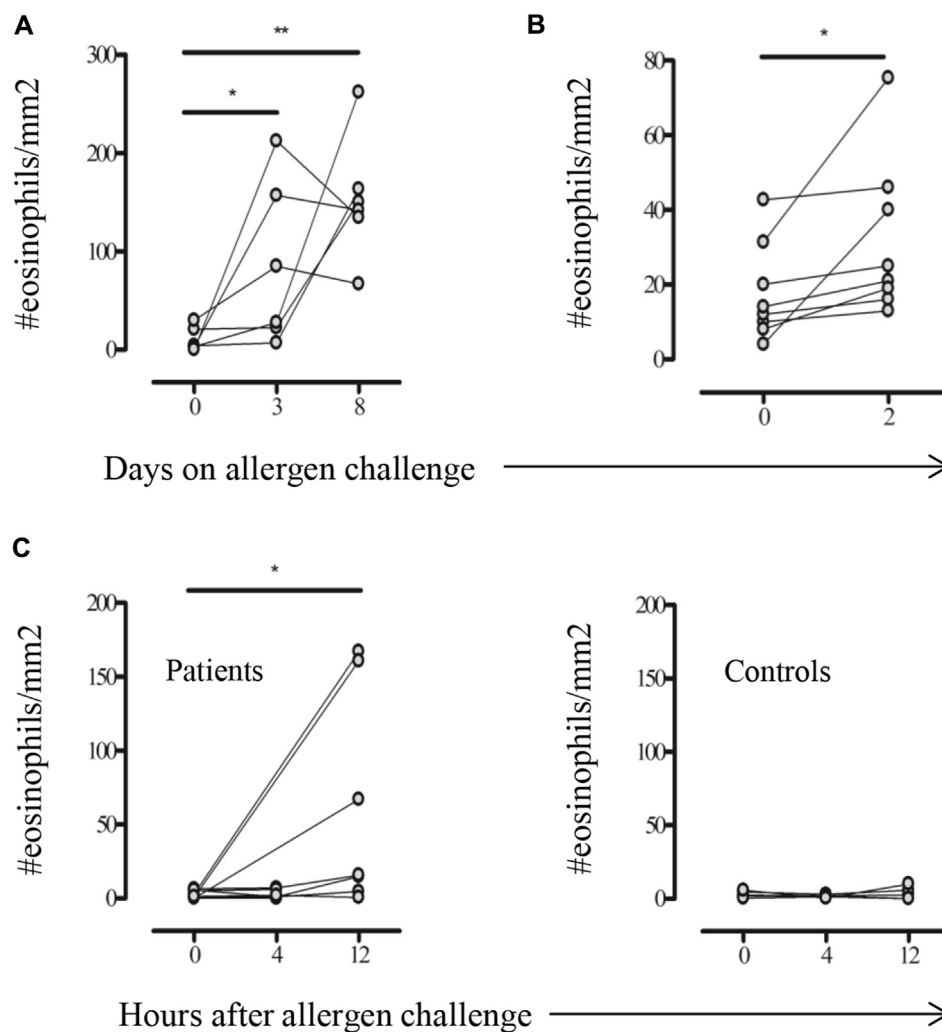


FIG E4. All provocation protocols induced accumulation of mucosal eosinophils in challenged patients but not in nonatopic control subjects. **A**, Density of eosinophils at baseline and after 3 and 8 days of challenge in patients from cohort I. **B**, Density of eosinophils at baseline and after 2 days of challenge in patients from cohort IV. **C**, Density of eosinophils at baseline and 4 and 12 hours after challenge in patients (*left*) and control subjects (*right*) from cohort II. * $P < .05$ and ** $P < .01$.

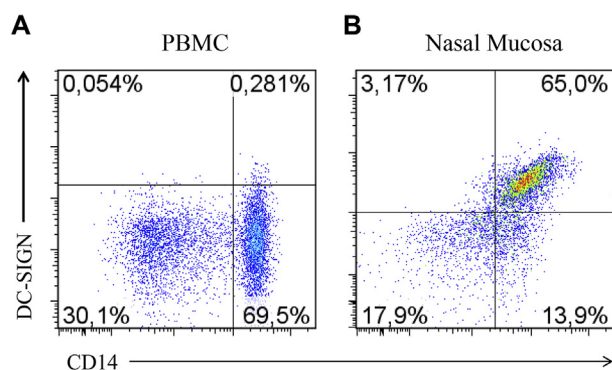


FIG E5. DC-SIGN is expressed on nasal mucosal macrophages but not blood monocytes. Representative dot plots show staining for CD14 and DC-SIGN on PBMCs (**A**) and cells released from nasal mucosal specimens (**B**) after enzyme digestion. Cells were pregated as live cells expressing CD45 and HLA-DR. The percentage of CD45⁺HLA-DR⁺ cells positive or negative for DC-SIGN and/or CD14 are given. Figures are representative of 3 donors.

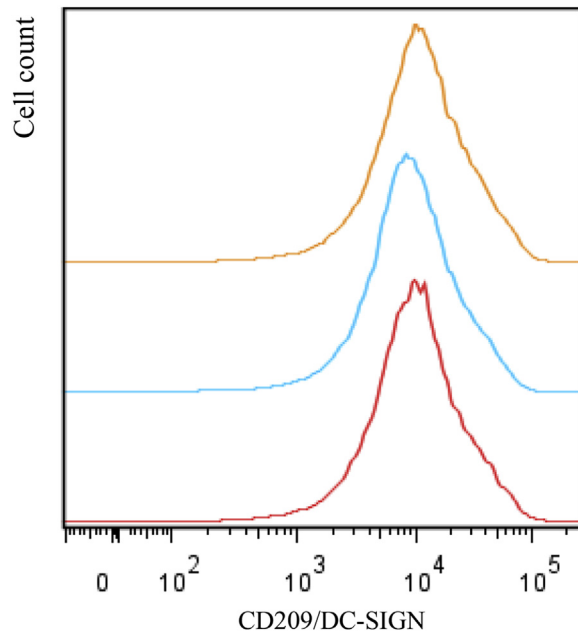


FIG E6. Treatment with pollen extracts does not affect DC-SIGN expression in moDCs, as assessed by using flow cytometry: *orange, Betula verrucosa* (birch); *blue, Phleum pratense* (timothy grass); *red, untreated cells*.

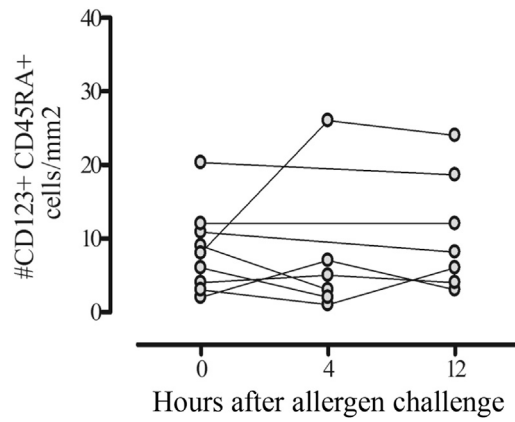


FIG E7. Density of pDCs does not increase in the nasal mucosa during the first hours after allergen challenge (cohort II). Density of CD123⁺CD45RA⁺ cells at baseline and 4 and 12 hours after challenge in allergic patients.

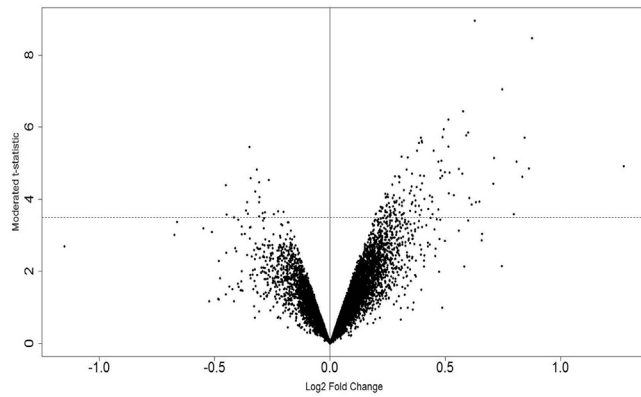


FIG E8. Identification of differentially expressed genes in TOPRO-1⁺CD19⁺CD3⁺CD45⁺HLA-DR⁺ cells after *in vivo* challenge (cohort III). Data are plotted as a volcano plot along axes of differential expression magnitude ($\log_2$ fold change) and versus the moderated t statistic.

TABLE E1. Antibodies used for flow cytometry and cell sorting

Antibody	Vendor	Clone	Fluorochrome	Use in the study
CD3	Life Technologies	UCHT1	PE	Cell sorting
CD14	BioLegend, San Diego, Calif	HCD14	Pacific Blue	Flow cytometry
CD19	BD Biosciences	HIB19	FITC	Cell sorting
CD45	BD Biosciences	2D1	APC-H7	Cell sorting
CD45	BioLegend	HI30	APC-Cy7	Flow cytometry
DC-SIGN	BD Biosciences	DCN46	APC	Flow cytometry
HLA-DR	BD Biosciences	L243	PE-Cy7	Cell sorting/flow cytometry

APC, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin.

TABLE E2. Primary antibodies used for immune fluorescence staining

Antibody	Vendor	Clone	Isotype
CD1a	Dako, Glostrup, Denmark	NA1/34	Mouse IgG _{2a}
CD1b	Gift from R. Blumberg	4A76	Mouse IgG _{2a}
CD3	BioProbe, Rüschlikon, Switzerland	RIV9	Mouse IgG ₃
CD3	Dako	AO452	Rabbit IgG
CD4	Novocastra, Newcastle, United Kingdom	IF6	Mouse IgG ₁
CD8	Becton Dickinson	SK1	Mouse IgG ₁
CD14	LifeSpan Biosciences, Seattle, Wash	18D11	Mouse IgG ₁
CD45RA	BD Biosciences	HI100	Mouse IgG _{2b}
CD123	BD Biosciences	7G3	Mouse IgG _{2a}
DC-SIGN	Gift from C. Westin	526.111	Mouse IgG _{2a}
GATA3	Santa Cruz Biotechnology, Dallas, Tex	SC-268	Mouse IgG ₁
HLA-DR	BD Biosciences	L243	Mouse IgG _{2a}
HLA-DR	Dako	TAL1B5	Mouse IgG ₁
Ki-67	Dako	MIB-1	Mouse IgG ₁
Major Basic Protein	ImmunoGen, Waltham, Mass	BMK-13	Mouse IgG ₁
L-selectin	Becton Dickinson	SK-11	Mouse IgG _{2a}

TABLE E3. Secondary antibodies/molecules used for immune fluorescence staining

Vendor	Detecting antibody/molecule	Detected antibody/molecule	Label
SouthernBiotech, Birmingham, Ala	Goat	Mouse IgG ₁	Biotin
Thermo Fisher Scientific	Goat	Mouse IgG ₁	A488
SouthernBiotech	Goat	Mouse IgG ₁	Cy3
Life Technologies	Goat	Mouse IgG ₁	A555
SouthernBiotech	Goat	Mouse IgG _{2a}	Biotin
SouthernBiotech	Goat	Mouse IgG _{2a}	Cy3
Thermo Fisher Scientific	Goat	Mouse IgG _{2a}	A555
SouthernBiotech	Goat	Mouse IgG _{2b}	A488
Molecular Probes, Eugene, Ore	Goat	Mouse IgG ₃	A488
Life Technologies	Donkey	Rabbit IgG	A488
GE Healthcare (UK), Chalfont St Giles, United Kingdom	Streptavidin	Biotin	Cy2
Vector Laboratories, Burlingame, Calif	Streptavidin	Biotin	AMCA

TABLE E4. Top 10 canonical pathways and upstream regulators associated with the set of genes that were upregulated in TOPRO-1⁺CD19⁺CD3⁺CD45⁺HLA-DR⁺ cells after allergen challenge

No.	Canonical pathways	P value	No.	Upstream regulator	Activation state	Z score	P value
1	Granulocyte adhesion and diapedesis	.0021380	1	IL-13	Activated	3.807	8.81E-18
2	Agranulocyte adhesion and diapedesis	.0026915	2	IL-4	Activated	3.64	8.14E-14
3	Lipid antigen presentation by CD1	.0030200	3	LPS	Activated	2.209	6.67E-08
4	CCR3 signaling in eosinophils	.0056234	4	ALOX15	Inconsistent	1.029	.000000478
5	Synaptic long-term potentiation	.0064565	5	STAT6	Inconsistent	1.436	.00000255
6	Atherosclerosis signaling	.0066069	6	CD40LG	Inconsistent	−0.342	.0000155
7	Protein kinase A signaling	.0067608	7	Infliximab	Inhibited	−2	.0000239
8	Cardiac β-adrenergic signaling	.0089125	8	IL-33	Inconsistent	0.594	.0000256
9	Glutathione redox reactions II	.0093325	9	ADA	Inconsistent		.0000471
10	Semaphorin signaling in neurons	.0117490	10	IL-10	Inconsistent	−0.241	.0000601

Differentially expressed genes were identified and analyzed in Ingenuity Systems software. Upregulated and downregulated genes were analyzed together. Upstream regulators are only included when the activation state was predicted from Ingenuity Systems. The activation state can only be predicted when the direction of the gene expression changes are consistent with prior studies.