

Persistent Activation of Dendritic Cells after Resolution of Allergic Airway Inflammation Breaks Tolerance to Inhaled Allergens in Mice

Leonie S. van Rijt^{1,3}, Nanda Vos¹, Monique Willart^{1,2}, Femke Muskens¹, Paul P. Tak⁴, Corine van der Horst⁵, Henk C. Hoogsteden¹, and Bart N. Lambrecht^{1,2}

¹Department of Pulmonary Medicine, Erasmus Medical Center, Rotterdam, The Netherlands; ²Laboratory of Immunoregulation and Mucosal Immunology, Department of Respiratory Medicine, University Hospital Gent, Gent, Belgium; ³Department of Experimental Immunology and ⁴Division of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands; and ⁵Arthrogen B.V., Amsterdam, The Netherlands

Rationale: Polysensitization of patients who are allergic is a common feature. The underlying immunologic mechanism is not clear. The maturation status of dendritic cells (DCs) is considered to be important for priming naive T cells in the draining lymph nodes. We hypothesized that chronic airway inflammation can induce an enhanced maturation of airway DCs and facilitate subsequent priming to neoallergens.

Objectives: To investigate whether chronic airway inflammation could induce an altered activation of airway DCs in mice and whether this influences the development of allergic sensitization.

Methods: Balb/c mice were repeatedly challenged with DCs to induce a chronic airway inflammation. We evaluated (1) the induction of the main characteristic features of human asthma including persistent remodeling, (2) the maturation status of airway DCs 1 month after inflammation resolved, (3) whether this influences tolerance to inhaled neoallergen, and (4) what type of T helper response would be induced by DCs.

Measurements and Main Results: Airway DCs displayed a mature phenotype after complete resolution of airway eosinophilia. Inhalation of a neoallergen without any adjuvant was able to induce airway inflammation in postinflammation lungs but not in control lungs. One month after inflammation, airway DCs were able to induce Th2 polarization in naive T cells consistent with the up-regulation of the Th2 skewing molecules Ym1/2 and OX-40L compared with DCs of control airways.

Conclusions: This study provides evidence that sustained maturation of DCs after resolution of Th2-mediated inflammation can contribute to polysensitization.

Keywords: murine asthma model; remodeling; Th2; neosensitization; mucosal immunity

Polysensitization is a common feature of patients with allergies (1). Both human and animal studies support the hypothesis that sensitization to one allergen favors sensitization to other environmental allergens (2–4). The immunologic mechanisms involved in allergic polysensitization are complex and incompletely understood.

(Received in original form January 5, 2011; accepted in final form April 28, 2011)

Supported by Erasmus University Fellowship Grant (L.S.v.R.) and a Netherlands Organization for Scientific Research VIDI fellowship (B.N.L.).

Authorship credits: Conception and design: L.S.v.R., H.C.H., and B.N.L.; acquisition of data: N.V., M.W., F.M., P.P.T., and C.v.d.H.

Correspondence and requests for reprints should be addressed to Bart N. Lambrecht, M.D., Ph.D., Department of Respiratory Medicine, Laboratory of Immunoregulation and Mucosal Immunology, MRB1, University Hospital Gent, De Pintelaan 185, B9000 Gent, Belgium. E-mail: bart.lambrecht@ugent.be

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 184, pp 303–311, 2011

Originally Published in Press as DOI: 10.1164/rccm.201101-00190C on May 11, 2011

Internet address: www.atsjournals.org

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Many patients who are allergic develop a sensitization for multiple allergens in time. Little is known about the underlying mechanism of polysensitization.

What This Study Adds to the Field

This animal study demonstrates that airway dendritic cells obtain an activated phenotype during inflammation that persists after resolution of the inflammation. This activation of dendritic cells was sufficient to break inhalational tolerance to inhaled neoallergens.

Several animal models have shown that an ongoing inflammation facilitated neosensitization on the condition that the neoallergen was administered simultaneously with the original antigen, referred to as “collateral priming.” These studies demonstrated that Th2 polarization of the naive neoallergen-specific T cells was dependent on IL-4 production by already primed Th2 cells (5–8). It has been recognized that airway dendritic cells (DC) play an important role in the activation of naive CD4 T cells by the delivery of different signals needed for a polarized Th2 cell response (9). An additional, not explored, mechanism underlying neosensitization might be that inflammation leads to enhanced maturation of airway DC leading to enhanced adaptive immune responses to newly encountered antigens. It has been shown that airway DCs obtain a more mature phenotype during an acute airway inflammation (10, 11). The fate of these matured DC after resolution of inflammation is, however, unclear. The half-life of migrated DC to the draining lymph nodes is considered to be short but evidence indicates that in the inflammatory state airway DC might survive for months in the periphery maintaining a more mature phenotype (12, 13). If this applies to allergic airway inflammation this could account for an additional mechanism underlying polysensitization, besides collateral priming. Studies on the mechanisms involved in allergic sensitization during chronic airway inflammation as in human asthma are hampered, in almost all animal models, by a phenomenon referred to as “airway inflammation-related inhibition of disease.” In contrast to human chronic asthma, in ovalbumin (OVA) allergen-sensitized mice that are exposed repetitively to allergen aerosols, eosinophilic airway inflammation wanes over time, because of decreased numbers of myeloid DC and an inhibition of costimulatory molecule up-regulation on DCs (14–16). To circumvent this phenomenon, we modified and extended a DC-driven

asthma model, as previously described (11, 17), in which we sensitized and repeatedly challenged mice with intratracheally administered mature DC, which present proteins processed from the culture medium, to induce repeated Th2 immune responses, leading to the main characteristics of human chronic asthma, including airway remodeling and basement membrane thickening.

In this study we aimed to elucidate whether persistent maturation of airway DC after a period of chronic airway inflammation can lead to neosensitization. Some of the results of these studies have been previously reported in the form of abstracts (18, 19).

METHODS

Animals

Female Balb/c, Ly5.2 C57Bl/6, Ly5.1 C57Bl/6, and DO11.10 mice (6–8 wks) were housed under specific pathogen-free conditions at the animal facility of Erasmus MC, Rotterdam, The Netherlands, and experiments were approved by the animal ethical committee.

Asthma Model Using Adoptive Transfer of DCs and Challenge Protocols

DCs were cultured from bone marrow cells derived from naive mice as previously described (11). To induce airway inflammation, mice were sensitized at Day 0 and challenged at Days 14, 28, 42, and 56 by an intratracheal injection of 1×10^6 unpulsed DCs (17), as previously described (20). One group of mice was sensitized at Day 0 by 1×10^6 OVA pulsed DCs intratracheally. At Day 9 of culture, cells were pulsed overnight with 100 μ g/ml OVA (Worthington Biochemical Corp., Lakewood, NJ) as described previously (11) and challenged with 1% OVA aerosol (Sigma, Zwijndrecht, The Netherlands; grade III in phosphate-buffered saline [PBS]) for 30 minutes, starting on Day 10 every other day for 7 weeks (see Figure 1A for challenge protocol). Neosensitization was induced by intratracheal administration of 100 μ g OVA (Sigma, grade III) 1 month after the last DC challenge. Fourteen days later sensitization was read out by challenging mice with a 1% OVA aerosol challenge for 3 consecutive days. One day after the last aerosol mice were sacrificed. Bronchoalveolar lavage fluid (BALF) cell differentiation, analysis of Th2 cytokine production by lung draining lymph nodes, OVA-IgE levels in serum, and airway histology were performed as described previously (11, 21, 22). For a description, see the METHODS section in the online supplement.

Isolation of DCs from Postinflammation Airways and Coculture with DO11.10 T Cells

For purification of DCs, a pool of 10 lungs of mice challenged five times with unpulsed DCs followed by 1 month rest and 10 lungs of mice that received PBS as a control were digested as described in the online supplement. Suspensions were stained with CD11c-PETx-Red, Major Histocompatibility Complex (MHC)II-Allophycocyanin (APC), CD11b-peridinin chlorophyll cychrome 5.5 (PerCPCy5.5), B220 Phycoerythrin (PE)-Cy7, and CD3/CD19-APC-Cy7; diamidine-2-phenylindole (DAPI) was used to distinguish between live and dead. Anti-CD16/CD32 was used to avoid aspecific binding. DCs were identified as CD11c^{hi}, MHCII^{hi}, CD11b^{hi}, autofluorescence^{low}, B220^{neg}, and CD3/CD19^{neg} (purity, 97.9% from postinflammation airways and 97.6% from control airways). Sorted cells were pulsed overnight with 10 μ g/ml OVA (Worthington). DCs were carefully washed before coculture with DO11.10 T cells. OVA-specific T Cell Receptor transgenic (TCR tg) cells were collected from the lymphoid organs of naive 4–6 week old DO11.10 mice and CD4⁺ T cells were isolated using a negative isolation magnet activated cell sorting kit following the manufacturer's instructions (94.9% pure) and stained with carboxy fluorescein diacetate succinimideylester (CFSE) (Invitrogen, Breda, The Netherlands) as previously described (20). The 1×10^6 T cells were cocultured with 1×10^4 pulsed DCs. After 3 days, cells were analyzed as previously described and supernatant was stored at -20°C for determination of IL-2, IL-4, IL-5, and IFN- γ levels by ELISA (20).

DC Phenotyping from Postinflammation Airways

For phenotyping DCs from postinflammation airways, DCs from postinflammation and control airways were stained as described previously in combination with PE-labeled CD80, CD86, CD40, intracellular adhesion molecule (ICAM)-1, inducible T-cell costimulator ligand, or the corresponding isotype control. Expression was analyzed by flow cytometry. Expression of Th2 skewing molecules, YM1/2 and OX40L, was determined by quantitative polymerase chain reaction, relative to the expression of household gene ubiquitin C. For more details see the online supplement.

Differentiation Assay of Naive OVA-Specific T Cells *In Vivo*

To investigate differentiation of naive T cells *in vivo* after chronic inflammation (1 month after the last unpulsed DC challenge), naive CFSE-labeled DO11.10 T cells were adoptively transferred intravenously. One day later a neoallergen (100 μ g OVA; Worthington) was instilled intratracheally in postinflammation or noninflammation mice. Three days after OVA administration, mice were killed and draining lymph nodes of the lung were analyzed for Th2 cytokine profiles of the adoptively transferred DO11.10 CD4⁺ T cells by intracellular staining and flow cytometry. Lymph nodes were activated in 10 μ g/ml anti-CD3 (kindly provided by L. Boon) coated wells for 4 hours in the presence of golgi stop (10 μ g/ml BD L51-2092KZ) at 37°C . Cells were washed thoroughly and stained for KJ-1.26-TC, IL-4 PE, IL-5 PE, IL-10 APC, biotinylated IL-13, or IFN- γ APC. Biotinylated antibodies were detected by a secondary staining with streptavidin-PE-CY7. The percentage of cytokine-positive T cells of total DO11.10 T cells was determined by Flowjo (Treestar, Ashland, OR).

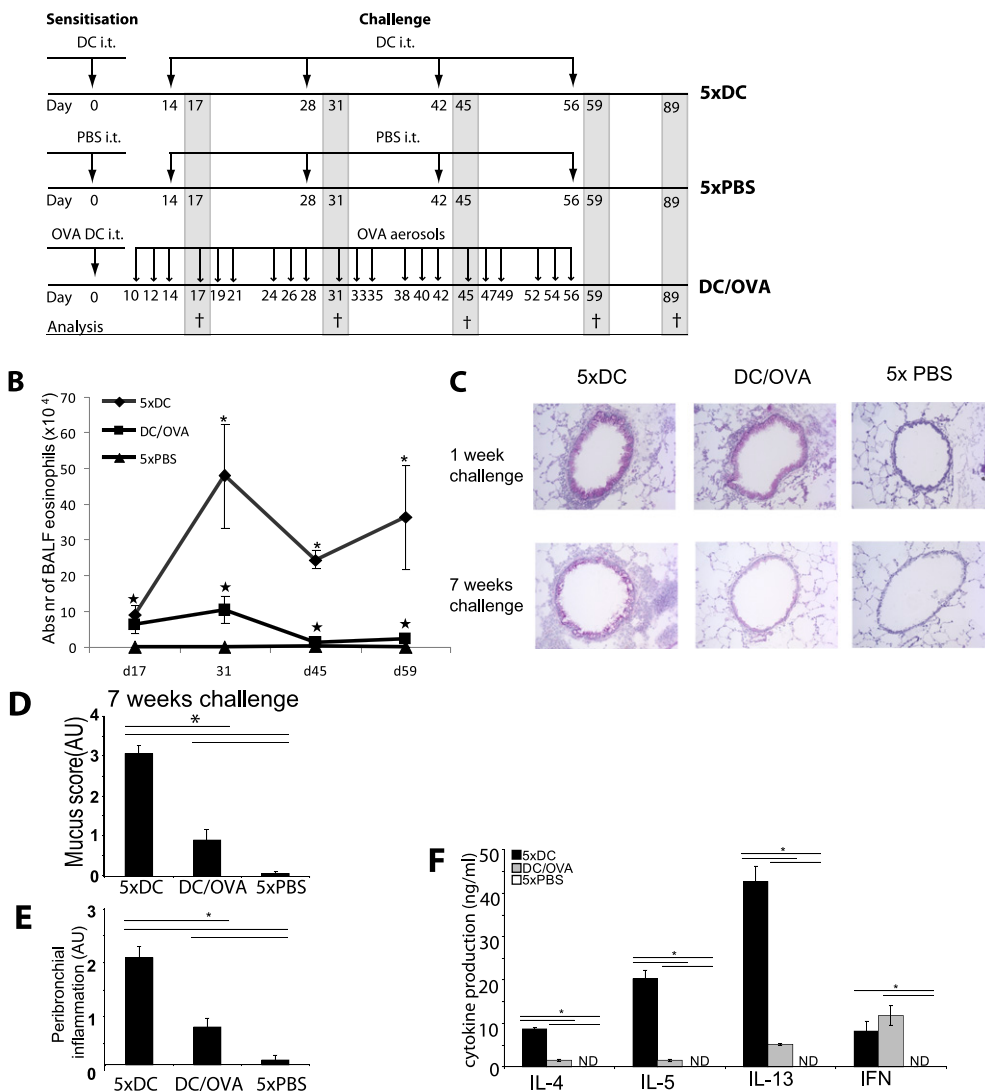
Statistical Analysis

For statistical analysis, Kruskal-Wallis one-way and the Mann-Whitney *U* test were performed. Differences were considered to be significant at a *P* value of less than 0.05. Each experiment was repeated two to six times with 5–10 mice per group.

RESULTS

Repeated Intratracheal Administration of DCs Induces a Persistent Th2 Response

Previously, others have shown that repetitive administration of inhaled soluble antigens, such as OVA, over weeks in OVA-alum sensitized mice or rats leads to progressive decline in the degree of airway inflammation caused by a state of immune tolerance in which the function of airway DCs is suppressed via induction of Treg cells (14–16, 23–28). We hypothesized that this down-regulation would not occur when mature DCs were used to induce sensitization and to challenge the mucosal immune system of the lung. *In vitro* cultured DCs were adoptively transferred to the airways by an intratracheal instillation. Although the DCs were not pulsed with a specific antigen, protein antigens contained in the fetal calf serum in the culture medium are likely to be presented and could act as allergen. We and others have previously reported that repeated intratracheal injection of allergen-pulsed bone marrow derived DCs obtained from these cultures can indeed be used to generate allergic-type acute airway inflammation and bronchial hyperreactivity in the absence of any added protein antigen, such as OVA (11, 17, 29). For comparison, we also immunized another group of Balb/c mice to one single intratracheal injection of OVA-pulsed bone marrow derived DCs followed by 7 weeks of OVA aerosol (DC-OVA; Figure 1A). The degree of inflammation induced was read-out by counting the number of recovered BALF eosinophils 3 days after the last DC challenge or OVA aerosol (Days 17, 31, 45, and 59) during the challenge period. Both groups were compared with a PBS control group that received PBS intratracheal every 2 weeks for five times in total. The number of recruited eosinophils (Figure 1B) was similar after

A Study Design:

the first unpulsed DC challenge or 1 week of OVA aerosols (Day 17), indicating that a similar degree of Th2 priming was induced by each experimental procedure. Both groups had a significantly higher recruitment of eosinophils compared with the PBS group. However, from 3-week OVA aerosol challenge onward, the recruitment of eosinophils to the airways decreased, up to a decrease of 63% at Week 7 (Day 59) compared with the first week of aerosols in the OVA-DC-sensitized mice. In mice repetitively challenged with unpulsed DCs the number of recruited BALF eosinophils increased by 400%. In concordance, histologic analysis of lung sections revealed that peribronchial inflammatory infiltrates and goblet cell hyperplasia were similar after the first week of challenge in both sensitization regimens. Progressively these features disappeared in the OVA-DC immunized group on repetitive OVA aerosols, yet were increased in mice receiving repetitive unpulsed DC challenges (Figure 1C and unpublished data). Blinded quantification of goblet cell hyperplasia (mucus score Figure 1D) and the degree of peribronchial inflammation (Figure 1E) using a previously established scoring system (21, 22) after 7 weeks of challenge substantiated these findings. Because eosinophilic airway inflammation can result from an adaptive Th2 response to inhaled antigen, we determined the type and levels of

Figure 1. Induction of chronic airway inflammation by repeated dendritic cell (DC) administrations to the airways. (A) Experimental design: mice were sensitized with unpulsed DCs, ovalbumin (OVA) pulsed DCs, and challenged with resp. DC instillation in the airways every 2 weeks for four times ($5 \times$ DC) or OVA aerosols three times per week for 7 weeks (DC-OVA). As a control we included mice intratracheally (i.t.) exposed to phosphate-buffered saline (PBS) instead of DCs ($5 \times$ PBS). $\dagger$ Day of analysis. (B) After 1 (d 17), 3 (d 31), 5 (d 45), and 7 (d 59) weeks of challenge, the number of $CCR3^+MHCII^+CD19^-CD3^-SSC^{int}$ eosinophils was determined in bronchoalveolar lavage fluid (BALF) by flow cytometry. $*P < 0.05$ versus DC-OVA and $5 \times$ PBS; small star symbol, $P < 0.05$ versus $5 \times$ PBS. (C) After 1 (upper panels) and 7 weeks (lower panels) of challenge lungs were stained with periodic acid Schiff to determine the degree of mucus production and goblet cell hyperplasia. (D) Scoring confirmed that DC-challenged mice had an increased goblet cell hyperplasia after 7 weeks of challenge. (E) Scoring confirmed the presence of increased peribronchial inflammation after 7 weeks of DC challenge. (F) After 7 weeks of challenge, draining lymph nodes of the lungs were cultured for 4 days and Th2 cytokine production was measured in supernatant by ELISA. Data presented are the means $\pm$ SEM. $*P < 0.01$. ND = not detected.

cytokines produced in draining mediastinal lymph nodes of the airways, cultured in the presence of OVA or fetal calf serum (Figure 1F). Although in both groups Th2 cytokines were produced, after 7 weeks of challenge significantly more IL-4, IL-5, and IL-13 was produced in the DC-challenged compared with the OVA aerosol-challenged mice. Similar Th2 cytokine patterns were observed at 1, 3, and 5 weeks of challenge (data not shown). These results showed that repetitive intratracheal administration of unpulsed DC can induce a chronic eosinophilic airway inflammation without waning of the response and therefore this protocol was used in all the following experiments.

Repeated DC Instillations Lead to Persistent Remodeling in the Airways

To determine whether the chronic DC-driven asthma model resulted in persistent changes in the airways, we analyzed the airways for collagen deposition and increased airway smooth muscle volume 1 month (Day 89, see Figure 1A) after the last challenge. One month after the last challenge, all airway eosinophilia was resolved, as revealed by the presence of only $0.99\% \pm 0.26$ eosinophils in lavage fluid, compared with $29.6\% \pm 5.1$ 3 days after the last DC challenge ($P < 0.05$).

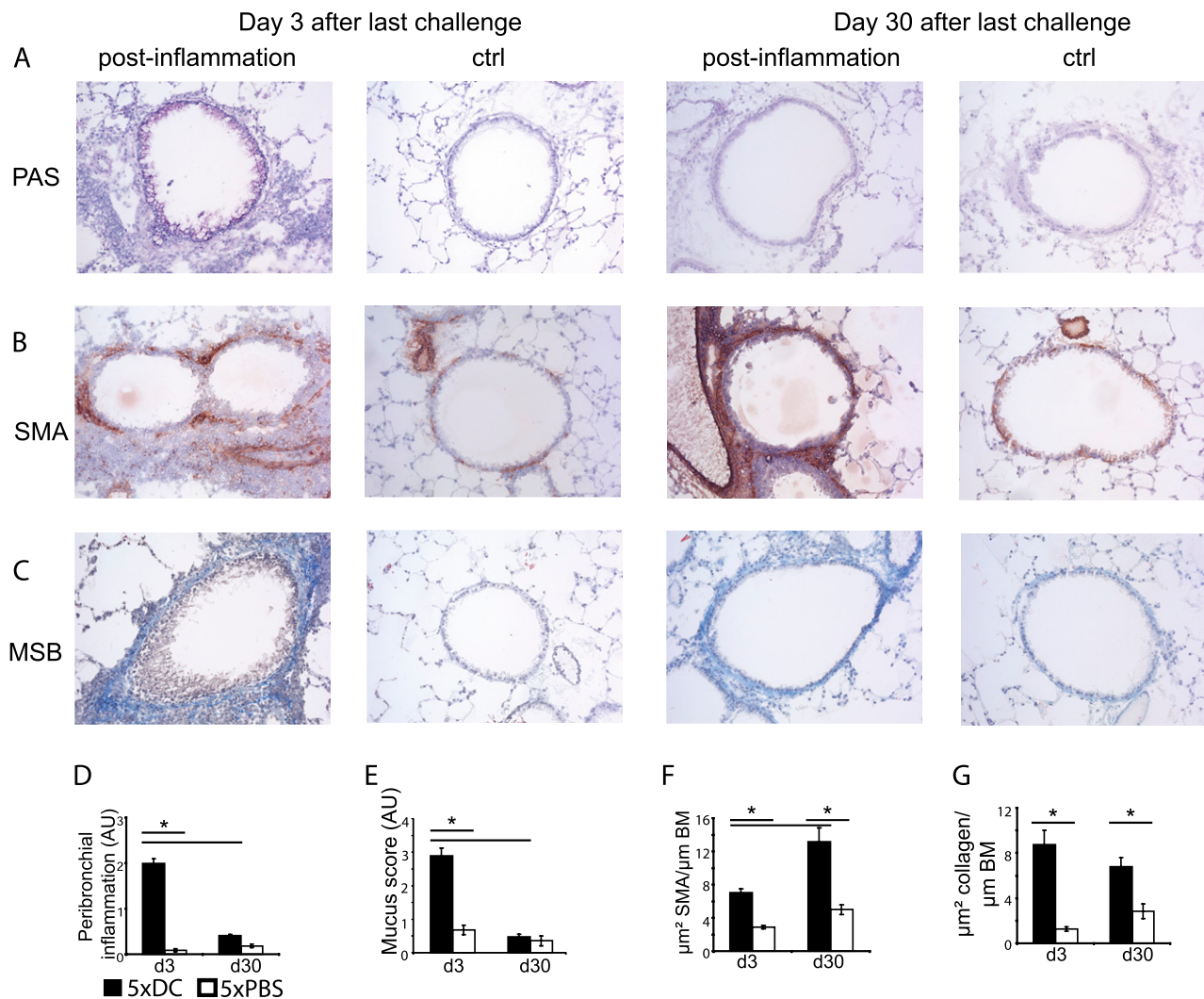


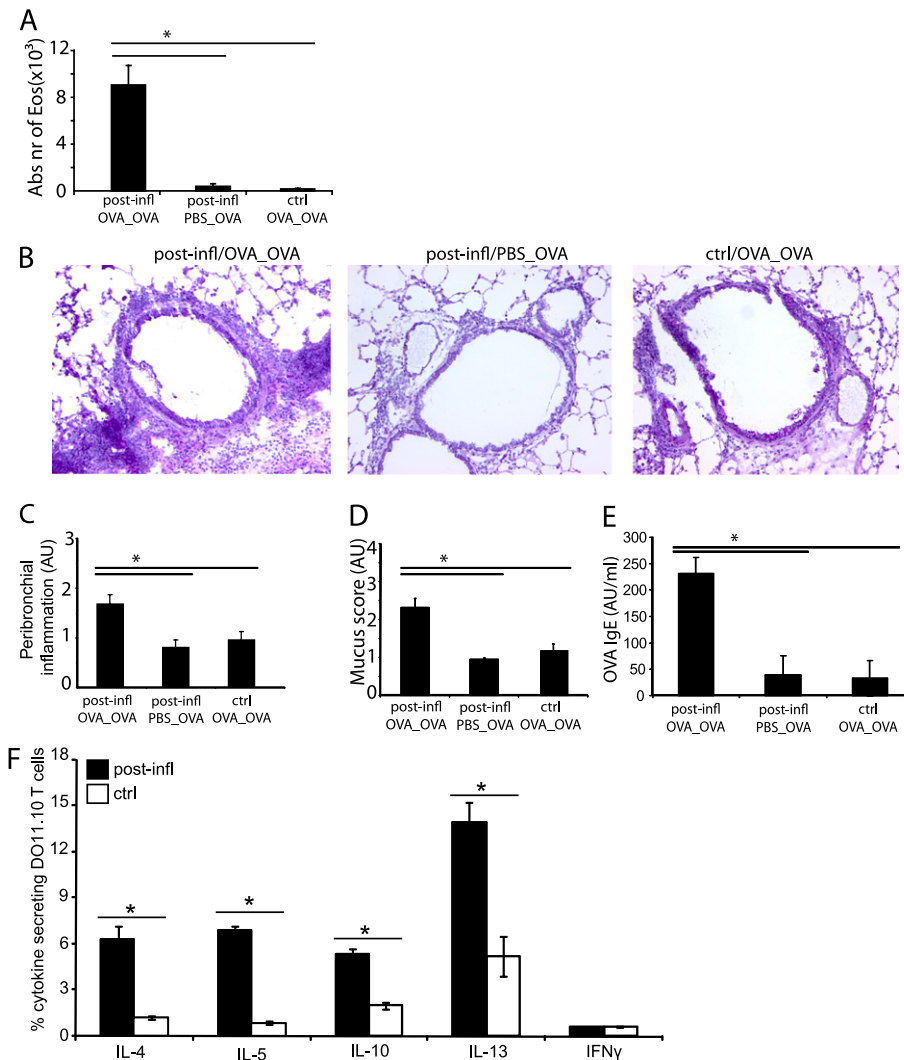
Figure 2. Induction of airway remodeling in mice receiving repetitive dendritic cells (DC) injections. Mice were challenged intratracheally with DCs (postinflammation) or as a control with phosphate-buffered saline (PBS) (ctrl) every 2 weeks for five times in total. Three or 30 days after the last challenge, lungs were stained for (A) mucus production (periodic acid Schiff [PAS]); (B) airway smooth muscle volume (SMA); and (C) collagen deposition (MSB). (D) Scoring confirmed that 30 days after the last challenge peribronchial inflammation and mucus production (E) were resolved completely (black bars represent mice that received $5 \times$ DC, white bars represent control mice that received $5 \times$ PBS). Quantification using an image analysis system confirmed the persistence of (F) increased airway smooth muscle volume per micrometer basal membrane (BM) and (G) collagen deposition per micrometer basal membrane 3 and 30 days after challenge. $*P < 0.05$.

Balb/c mice, which were challenged five times with DCs ($5 \times$ DC, 1 month rest) and had a subsequent rest period of 1 month are referred to as “postinflammation” mice and mice treated with PBS instead of DCs are referred to as “control” mice ($5 \times$ PBS, 1 month rest) from here on. Histologic analysis showed that peribronchial infiltrates and enhanced mucus production that were present at Day 3 after the last challenge were completely resolved after 1 month (Figure 2A, quantified in Figures 2D and 2E). In contrast, structural changes, such as increased peribronchial airway smooth muscle volume (Figure 2B, quantified in Figure 2F) and subepithelial collagen deposition (Figure 2C, quantified in Figure 2G), were still present in post-inflammation mice after 1 month rest and were significantly increased compared with control airways.

Chronic Airway Inflammation Promotes De Novo Th2 Sensitization to Inhaled Innocuous Protein Antigen

To determine whether chronic airway inflammation could modulate the threshold of sensitization to a new inhaled protein

allergen, postinflammation or control mice received $100 \mu\text{g}$ of OVA or PBS avoiding the use of any adjuvant. Sensitization was read-out by challenging Balb/c mice with three OVA aerosols 2 weeks later. As reported previously, OVA challenge in OVA-treated control mice (without preceding inflammation) was not sufficient to recruit eosinophils to the airways (30). In postinflammation mice, however, this protocol induced a significant recruitment of eosinophils to the bronchoalveolar space (Figure 3A). Postinflammation mice that received intratracheal PBS as a priming regimen and were subsequently OVA challenged did not develop any eosinophilia, illustrating it was not the OVA aerosol or trace amounts of LPS contained in it *per se* that triggered or recalled inflammation. Histologic analysis and quantification substantiated these findings and revealed increased peribronchial inflammatory infiltrates (Figure 3B, quantified in Figure 3C). Periodic acid Schiff staining on airway slides demonstrated that goblet cell hyperplasia was induced after OVA challenge in postinflammation mice but not in control mice



(quantified as mucus score in Figure 3D). To investigate whether sensitization to OVA occurred, OVA-specific IgE levels were measured in serum. In concordance, only in post-inflammation mice OVA priming without any adjuvant and subsequent challenging with OVA could induce a significant level of OVA IgE in serum but not in control mice. Post-inflammation mice that were primed with PBS and challenged with OVA did not produce significant levels of OVA-specific IgE (Figure 3E).

The presence of airway eosinophilia, goblet cell hyperplasia, and OVA-specific IgE were reminiscent of a Th2 response to OVA because these features are IL-5, IL-4/13, and IL-4 dependent, respectively. To determine whether naive OVA-specific T cells were polarized to Th2 cells, we performed an additional experiment in which we transferred a cohort of naive CFSE-labeled OVA-specific TCR Tg T cells in postinflammation mice and in control mice followed by intratracheal administration of a single dose of OVA. This cohort was stained for intracellular cytokine production in antigen-reactive T cells 3 days later in the mediastinal lymph nodes. In both groups a similar proliferation of OVA-specific T cells was observed but only in postinflammation mice; differentiation to IL-4 $^{+}$, IL-5 $^{+}$, IL-10 $^{+}$, and IL-13 $^{+}$ -producing cells occurred and not in the cohort that was transferred into control non-inflammation airways; whereas IFN- γ was not induced (Figure 3F) in both groups.

Figure 3. Intratracheal administration of allergen without adjuvant induces *de novo* sensitization in postinflammation mice. (A) Postinflammation mice ($5 \times$ dendritic cell [DC] intratracheal with subsequent 1 mo rest) were sensitized intratracheally with 100 μ g ovalbumin (OVA) ($5 \times$ DC-OVA_OVA) in the absence of any adjuvant or as a control with phosphate-buffered saline (PBS) ($5 \times$ DC-PBS_OVA). Control mice were instilled with OVA ($5 \times$ PBS-OVA_OVA). Ten days later all groups were challenged with OVA aerosols for 3 consecutive days. The number of recruited eosinophils to the bronchoalveolar compartment was determined by flow cytometry. (B) Lung slides of mice described under A were stained with periodic acid Schiff to determine mucus production and formation of peribronchial inflammatory infiltrates. (C and D) Scoring confirmed formation of peribronchial inflammatory infiltrates and increased goblet cell hyperplasia only in mice with postinflammation airways and not in normal airways after OVA exposure and subsequent OVA challenge. (E) OVA IgE levels in serum. (F) Postinflammation mice or control mice received a naive cohort of OVA T Cell Receptor transgenic cells. One day later 100 μ g OVA was intratracheally instilled. Intracellular staining for IL-4, IL-5, IL-10, IL-13, and IFN- γ demonstrated more Th2 cytokine-positive cells in the cohort that were adoptively transferred in postinflammation mice compared with control mice. $*P < 0.05$.

Airway DCs Have an Activated Phenotype after Resolution of Eosinophilia Associated with an Enhanced Sentinel Function

Because DC are crucial for the outcome of immune responses, we hypothesized that DC could maintain their matured phenotype after resolution of eosinophilic airway inflammation. We and others have previously shown that DC obtain a more mature phenotype during acute airway inflammation, but it is unclear whether the DC population returns to the steady state phenotype or whether a mature phenotype is maintained after resolution of chronic eosinophilia (10, 11, 13).

In postinflammation or control mice the number of CD11b $^{+}$ CD11c $^{+}$ MHCII $^{+}$ lung DCs was not significantly different (each representing 0.2% viable CD11b $^{+}$ CD11c $^{+}$ MHCII $^{+}$ cells of total lung cells). Phenotyping by flow cytometry revealed that the recipient lung CD11b $^{+}$ subset of DCs purified from postinflammation mice had higher expression levels of CD80, CD86, CD40, and ICAM-1 compared with DCs purified from control mice (Figure 4A, quantified in Figure 4B). For comparison, levels of costimulatory molecules were also determined on other CD11c $^{+}$ lung cells including alveolar macrophages, CD11b $^{-}$ DC subsets, and pDCs (31). None of these subsets demonstrated the increase in costimulatory molecules seen in CD11b $^{+}$ CD11c $^{+}$ DCs (data not shown). To exclude that bone marrow derived DCs that were injected contaminated the

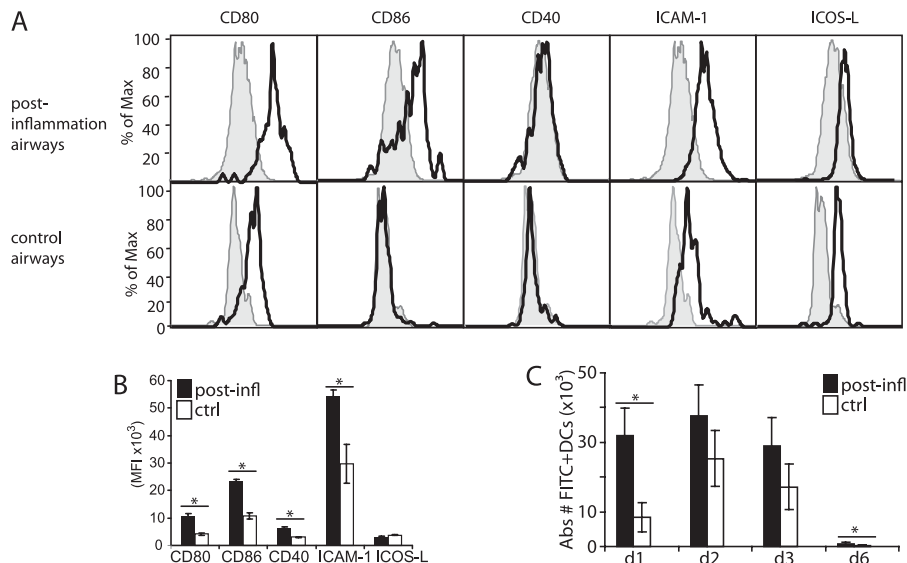


Figure 4. Airway dendritic cells (DC) have a persistently activated phenotype and enhanced sentinel function after chronic inflammation. (A) Expression of surface molecules involved in T cell interaction or adhesion by CD11b⁺CD11c⁺MHCII⁺ DCs in postinflammation mice (5 × DC intratracheal with 1 mo of rest) and in control mice (5 × phosphate-buffered saline [PBS] intratracheal with 1 mo rest). (B) Expression of surface molecules on airway DC was quantified as the average mean fluorescent intensity (MFI) ± SEM per group. (C) Postinflammation mice or control mice (resp. 5 × DCs or 5 × PBS intratracheal with 1 mo rest) were instilled intratracheally with fluorescein isothiocyanate (FITC) labeled OVA. Twenty-four, 48, 72, and 144 hours later, the presence of FITC-positive migrating DCs in lung draining lymph nodes were analyzed by flow cytometry. ICAM = intracellular adhesion molecule; ICOS-L = inducible T-cell costimulator ligand. *P < 0.05.

phenotyped population, we performed a pilot experiment in C57Bl/6 mice with DC expressing the congenic Ly5.1 marker, allowing us to discriminate any residual bone marrow derived DCs from recipient Ly5.2 lung DCs. No Ly5.1⁺ DCs were detectable 1 month after the last challenge (data not shown) making it unlikely that the observed phenotype was caused by residual transferred DCs.

We next examined whether airway DC migration to the draining lymph nodes was enhanced in postinflammation mice. We therefore administered intratracheally fluorescently labeled OVA to control (5 × PBS, 1 mo rest) or postinflammation mice (5 × DC, 1 mo rest). Twenty-four hours after administration, significant more OVA–fluorescein isothiocyanate–positive laden DCs could be found in the mediastinal lymph nodes of postinflammation mice compared with control mice (Figure 4C). To investigate whether the enhanced migration was compensated at later time points also 48, 72, and 144 hours after allergen administration, lung draining lymph nodes were analyzed. At later time points the number of migrated DCs did not differ significantly, suggesting both a fastened and enhanced DC migration in postinflammation airways.

Airway DCs in Postinflammation Lungs Are Sufficient to Induce Th2 Polarization in Naive Antigen-specific T Cells *In Vitro*

Although these experiments strongly suggested a hyperactivated DC system as the culprit for Th2 induction to protein antigens in postinflammation airways, the presence of costimulatory molecules and enhanced migration is not enough to conclude that airway DCs induce a productive effector Th2 response (32). These experiments also do not exclude that the postinflammation lung milieu could facilitate neosensitization without the use of any adjuvant, for instance by the presence of cytokines. To address whether lung DCs from postinflammation mice were capable to induce Th2 differentiation in naive antigen-specific CD4⁺ T cells without the presence of the postinflammation airway milieu, we flow-sorted DCs from digested lungs from a pool of 10 postinflammation or control mice, and exposed them to a dose of 10 µg/ml OVA *in vitro*. DCs were thoroughly washed and subsequently cocultured with naive CFSE-labeled OVA TCR Tg CD4⁺ T cells. As expected based on their higher level

of costimulatory molecule expression, airway DCs from postinflammation mice induced more proliferation compared with control airway DCs (Figure 5A: 8.9% vs. 5% of all T cells). To explore whether DCs from postinflammation airways were able to induce a polarized cytokine response in dividing T cells, the levels of IL-2, IL-4, IL-5, and IFNγ were analyzed in supernatant. T cells stimulated with DCs isolated from postinflammation airways secreted IL-2, IL-4, IL-5, and IFNγ, whereas DCs sorted from control mice only induced a threefold lower IL-2 and IFNγ production in responding T cells (Figure 5B).

To study whether chronic inflammation can have persistent effects on DC-expressed genes involved in Th2 skewing, total RNA was isolated from sorted airway DC and compared for expression of Ym1/2 and OX40L. Recently, it was shown that in allergic airways Ym1/2 expression in DCs is up-regulated in an IL-13–dependent way during airway inflammation and leads to enhanced Th2 cytokine production in CD4⁺ T cells (33). DC expression level of Ym1/2 was 10 times increased in postinflammation airways compared with DCs from control airways. Next, we analyzed expression of OX-40L (34), recently described to skew naive T cells into Th2 differentiation. Postinflammation airway DCs also expressed relatively higher levels of OX-40L compared with DCs from control airways (Figure 5C).

Collectively, our data show that DCs remain in a pro-Th2 state after resolution of eosinophilic airway inflammation, suggesting that the persistent activation of DCs could be involved in the underlying process of polysensitization in chronic allergic asthma.

DISCUSSION

The initiation of a Th2 immune response is largely dependent on cytokine milieu, the microenvironment, and the maturation status of the antigen-presenting DC. In this study we focused on the role of airway DCs in neosensitization in asthmatic airways. Our study shows that in postinflammation airways in which acute eosinophilia had resolved, CD11b⁺/CD11c⁺ airway DCs did not return to their steady-state phenotype but persistently expressed high levels of costimulatory molecules, such as CD80, CD86, and ICAM-1, and had enhanced expression of the Th2 skewing molecules OX-40L and Ym1/2. This enhanced maturation status of airway DC facilitated neosensitization up to at least 1 month after inflammation. Maturation of DCs is often considered

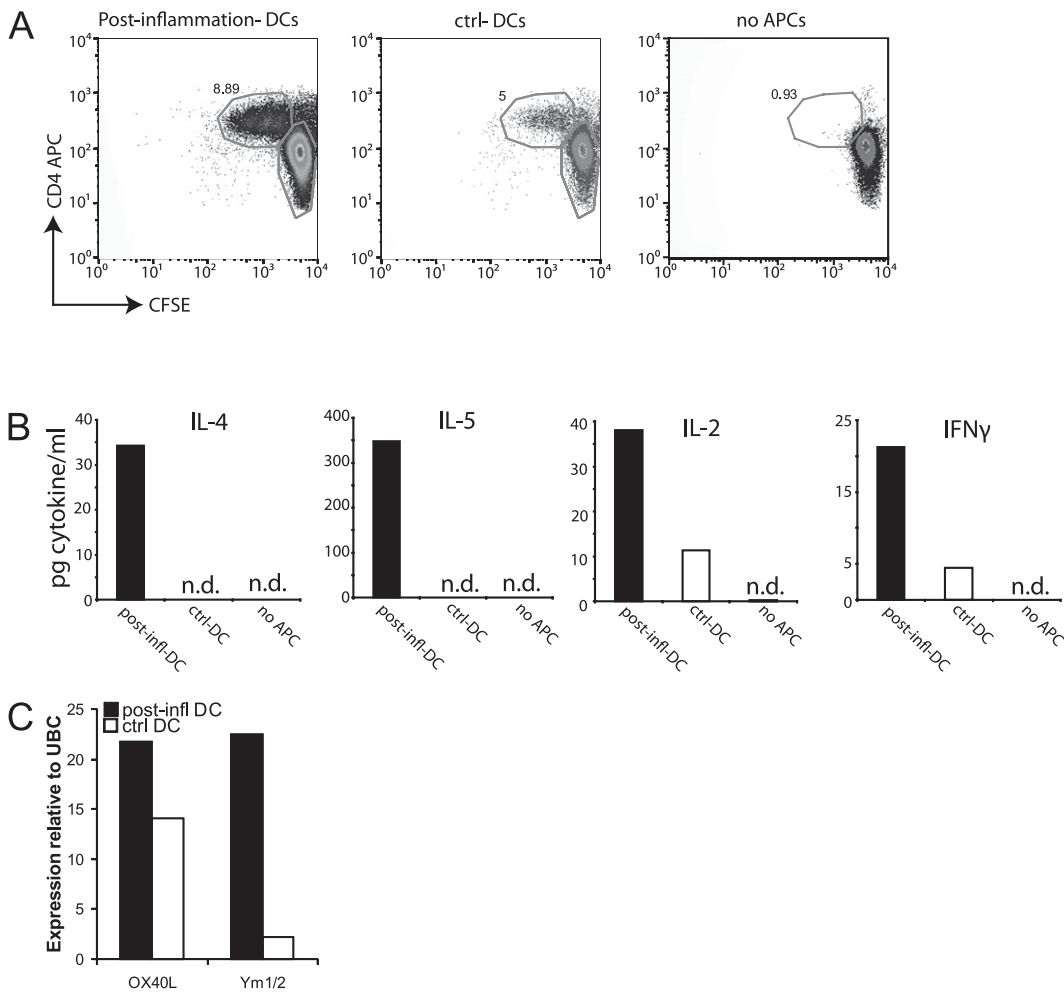


Figure 5. Postinflammation airway dendritic cells (DC) induce Th2 polarization in naive T cells, which is associated with expression of Th2 skewing molecules. (A) DCs were sorted as CD11c^{hi}, MHCII^{hi}, CD11b^{hi}, autofluorescent^{lo}, B220^{neg}, CD3^{neg}, CD19^{neg}, and pulsed overnight with ovalbumin (OVA) and subsequently cocultured with isolated carboxy fluorescein diacetate succimidylester⁺ CD4⁺ OVA T Cell Receptor transgenic T cells for 3 days in a ratio DC:T cell of 1:10. Proliferation plots are depicted of T cells activated by DCs from post-inflammation airways, control airways, or with no Allophycocyanin. (B) Levels of IL-4, IL-5, IL-2, and IFN- γ were determined in supernatant by ELISA. n.d. = not detected. (C) Real-time reverse transcriptase polymerase chain reaction was performed for Ym1/2 and OX40-L on isolated DCs from postinflammation airways (after 1 mo rest) or control (5 $\times$ PBS) airways. Fold increase in gene expression was calculated using housekeeping gene ubiquitin (UBC) for standardization. One representative experiment of two performed experiments is shown.

as a transition from antigen-capturing cells to antigen-presenting cells with an enhanced migratory capacity. In accordance with our results, it was, however, shown recently that mature DCs continue to process and present allergens (35).

In humans with allergic asthma, airway DC were shown to have a more mature phenotype compared with healthy control subjects (36). Additionally, monocyte-derived DCs from donors allergic to house dust mite express higher levels of CD86 and OX-40L when exposed to the allergen Der p 1 than healthy control DCs (37). However, in humans, the effect of an ongoing inflammation in the maintenance of a mature DC phenotype is difficult to determine. We are aware that a murine model has its limitations in the translation to the human allergic airway inflammation, but this model enabled us to study the question whether DCs remain more mature after resolution of the eosinophilic airway inflammation.

The finding of persistent DC activation after resolution of inflammation was much unexpected. Several mouse studies have demonstrated that antigen-matured DCs migrate to the draining lymph nodes and are replaced by immature DCs (38, 39). In OVA-challenged airways, the expression of costimulatory molecules like CD86 occurs only very transiently (24-h maximum) on respiratory tract DCs of sensitized rats, but is a requisite for mounting adaptive immune responses to OVA antigen (38). Others have shown that the main reason why prolonged OVA exposure fails to induce persistent eosinophilic inflammation is through down-regulation of DC function, associated with down-regulated expression of costimulatory molecules (14, 28).

The increased expression of costimulatory molecules does not explain why “postinflammation” airway DCs instructed naive T cells for differentiation into Th2 cells *in vivo* and *in vitro*. Previous studies in allergic airway inflammation models have demonstrated the expression of Th2 skewing molecules by DCs during acute inflammation. One month after inflammation, we observed an up-regulated expression of Ym1/2 (33) and OX-40L (34), compared with control DCs. This increased expression might support the Th2 skewing capacity of DCs after airway inflammation.

Our findings suggest that persistent activation of DCs induced by chronic allergic inflammation promoting sensitization to newly encountered antigens is a possible scenario for cumulative sensitizations in individuals. This scenario differs from the observation that the presence of an ongoing Th2 response creates a milieu that promotes Th2 responses to neoantigens, a process called “collateral priming” that was shown to depend on paracrine effects of IL-4 on Th2 priming (5). We do not believe this is the mechanism for our observations because collateral priming occurs under conditions where T cells are primed concomitantly by the same APC, unlikely to occur here in view of the long delay between the last DC administration and OVA exposure. Our data suggest that the window of opportunity to induce a Th2 response to inhaled neoallergen in allergic airways is not restricted within the narrow limits of activation of T cells to the first allergen.

We have not addressed mechanisms responsible for the sustained activation of airway DCs. Transient activation of lung

DCs is thought to result from brief contact with activated T cells, expressing CD40L (38). Chronic airway inflammation might lead to prolonged residence of DCs in the airways, possibly leading to prolonged encounters with activated T cells and persistent activation of DCs (12, 38). The persistent expression of ICAM-1 on airway DCs can contribute to long-lasting contacts with T cells (40). In support of this theory, we have preliminary data to show increased numbers of memory phenotype (CD44^{hi} CD69^{hi}) T cells clustering with DCs 1 month after the last challenge (data not shown). Memory and effector Th2 cells can persist in the airways up to at least 8 weeks after allergen challenge (12). Although contact with activated T cells might offer some explanation for our findings, several other possibilities exist. It could also be that deposition of altered extracellular matrix inside postinflammation airways profoundly alters the behavior or maturation of lung DCs, as proposed in the past (41, 42). The lack of LPS-free extracts of collagens and other extracellular matrix components hindered testing the direct effect of these components on DC maturation, an observation also reported by others (data not shown and [43]). However, besides this potentially activating effect on DCs, subepithelial basement membrane thickening might also dampen the immune response. Thickening of the lamina reticularis might provide a barrier to the effective sampling of the airway luminal contents by DCs, to shield-off the antigen from the immune system, akin to the situation in chronic parasitic infection or foreign body granulomas where fibrosis is also thought to encapsulate persistent antigens that cannot be destroyed by the immune system (44). Future experiments will have to elucidate this further. Another possibility that we have not yet explored is that activated structural cells of the remodeled airways, such as myofibroblasts, airway smooth muscle cells, or epithelial cells, release increased amounts of DC maturation cytokines, such as tumor necrosis factor- α , granulocyte-macrophage colony-stimulating factor, or Thymic Stromal Lymphopoietin (9, 45). However, an experiment in which we irradiated postinflammation mice and reconstituted them with donor bone marrow did not support this hypothesis because the donor-derived airway DCs displayed a similar phenotype as in control airways (data not shown). This suggested that structural changes are not exclusively responsible for the persistent maturation of airway DC, and a hematopoietic component could be involved. In future murine studies it will be of interest to investigate the underlying mechanism of persistent maturation of airway DCs after allergic airway inflammation and whether this described mechanism in mice can be translated to human chronic asthma. Further studies in humans are needed.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Prigione I, Morandi F, Tosca MA, Silvestri M, Pistoia V, Ciprandi G, Rossi GA. Interferon-gamma and IL-10 may protect from allergic polysensitization in children: preliminary evidence. *Allergy* 2010;65:740–742.
- Des Roches A, Paradis L, Menardo JL, Bouges S, Daures JP, Bousquet J. Immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract. VI. Specific immunotherapy prevents the onset of new sensitizations in children. *J Allergy Clin Immunol* 1997;99:450–453.
- Purello-D'Ambrosio F, Gangemi S, Merendino RA, Isola S, Puccinelli P, Parmiani S, Ricciardi L. Prevention of new sensitizations in monosensitized subjects submitted to specific immunotherapy or not. A retrospective study. *Clin Exp Allergy* 2001;31:1295–1302.
- Pajno GB, Barberio G, De LF, Morabito L, Parmiani S. Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study. *Clin Exp Allergy* 2001;31:1392–1397.
- Eisenbarth SC, Zhadkevich A, Ranney P, Herrick CA, Bottomly K. IL-4-dependent Th2 collateral priming to inhaled antigens independent of Toll-like receptor 4 and myeloid differentiation factor 88. *J Immunol* 2004;172:4527–4534.
- Dittrich AM, Chen HC, Xu L, Ranney P, Connolly S, Yarovinsky TO, Bottomly HK. A new mechanism for inhalational priming: IL-4 bypasses innate immune signals. *J Immunol* 2008;181:7307–7315.
- Perona-Wright G, Mohrs K, Mayer KD, Mohrs M. Differential regulation of IL-4R α expression by antigen versus cytokine stimulation characterizes Th2 progression in vivo. *J Immunol* 2010;184:615–623.
- Cadot P, Meyts I, Vanoirbeek JA, Vanaudenaerde B, Bullens DM, Ceuppens JL. Sensitization to inhaled ryegrass pollen by collateral priming in a murine model of allergic respiratory disease. *Int Arch Allergy Immunol* 2010;152:233–242.
- Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;8:193–204.
- Vermaelen K, Pauwels R. Accelerated airway dendritic cell maturation, trafficking, and elimination in a mouse model of asthma. *Am J Respir Cell Mol Biol* 2003;29:405–409.
- van Rijt LS, Jung S, KleinJan A, Vos N, Willart M, Duez C, Hoogsteden HC, Lambrecht BN. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 2005;201:981–991.
- Julia V, Hessel EM, Malherbe L, Glaichenhaus N, O'Garra A, Coffman RL. A restricted subset of dendritic cells captures airborne antigens and remains able to activate specific T cells long after antigen exposure. *Immunity* 2002;16:271–283.
- Dahl ME, Dabbagh K, Liggitt D, Kim S, Lewis DB. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol* 2004;5:337–343.
- Koya T, Kodama T, Takeda K, Miyahara N, Yang ES, Taube C, Joetham A, Park JW, Dakhama A, Gelfand EW. Importance of myeloid dendritic cells in persistent airway disease after repeated allergen exposure. *Am J Respir Crit Care Med* 2006;173:42–55.
- Niu N, Le Goff MK, Li F, Rahman M, Homer RJ, Cohn L. A novel pathway that regulates inflammatory disease in the respiratory tract. *J Immunol* 2007;178:3846–3855.
- Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, Sly PD, Holt PG. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J Exp Med* 2006;203:2649–2660.
- van Rijt LS, Vos N, Willart M, KleinJan A, Coyle AJ, Hoogsteden HC, Lambrecht BN. Essential role of dendritic cell CD80/CD86 costimulation in the induction, but not reactivation, of TH2 effector responses in a mouse model of asthma. *J Allergy Clin Immunol* 2004;114:166–173.
- van Rijt L, Vos N, Muskens F, Willart M, Hoogsteden H, Lambrecht B. Chronic airway inflammation promotes de novo sensitisation to inhaled allergens by persistent activation of dendritic cells [abstract 188]. *Allergy* 2009;64:88.
- van Rijt LS, Vos N, Willart M, Muskens F, Hoogsteden H, Lambrecht B. Chronic airway inflammation promotes de novo sensitization to inhaled allergen by persistent activation of dendritic cells [abstract 859]. *J Allergy Clin Immunol* 2009;123:S223.
- Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* 2000;106:551–559.
- Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;282:2261–2263.
- Curtis JL, Byrd PK, Warnock ML, Kaltreider HB. Requirement of CD4-positive T cells for cellular recruitment to the lungs of mice in response to a particulate intratracheal antigen. *J Clin Invest* 1991;88:1244–1254.
- Kumar RK, Foster PS. Modeling allergic asthma in mice: pitfalls and opportunities. *Am J Respir Cell Mol Biol* 2002;27:267–272.
- Schramm CM, Puddington L, Wu C, Guernsey L, Gharace-Kermani M, Phan SH, Thrall RS. Chronic inhaled ovalbumin exposure induces

- antigen-dependent but not antigen-specific inhalational tolerance in a murine model of allergic airway disease. *Am J Pathol* 2004;164:295–304.
25. Swirski FK, Sajic D, Robbins CS, Gajewska BU, Jordana M, Stampfli MR. Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor. *J Immunol* 2002;169:3499–3506.
26. Singh A, Carson WF, Secor ER Jr, Guernsey LA, Flavell RA, Clark RB, Thrall RS, Schramm CM. Regulatory role of B cells in a murine model of allergic airway disease. *J Immunol* 2008;180:7318–7326.
27. Cui ZH, Joetham A, Aydinoglu MK, Hahn YS, Born WK, Gelfand EW. Reversal of allergic airway hyperreactivity after long-term allergen challenge depends on gammadelta T cells. *Am J Respir Crit Care Med* 2003;168:1324–1332.
28. Van Hove CL, Maes T, Joos GF, Tournoy KG. Prolonged inhaled allergen exposure can induce persistent tolerance. *Am J Respir Cell Mol Biol* 2007;36:573–584.
29. Ostler T, Ehl S. A cautionary note on experimental artefacts induced by fetal calf serum in a viral model of pulmonary eosinophilia. *J Immunol Methods* 2002;268:211–218.
30. de Heer HJ, Hammad H, Soullie T, Hijdra D, Vos N, Willart MA, Hoogsteden HC, Lambrecht BN. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 2004;200:89–98.
31. Geurts van Kessel CH, Willart MA, van Rijt LS, Muskens F, Kool M, Baas C, Thielemans K, Bennett C, Clausen BE, Hoogsteden HC, *et al.* Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *J Exp Med* 2008;205:1621–1634.
32. Reis e Sousa C. Dendritic cells in a mature age. *Nat Rev Immunol* 2006;6:476–483.
33. Cai Y, Kumar RK, Zhou J, Foster PS, Webb DC. Ym1/2 promotes Th2 cytokine expression by inhibiting 12/15(S)-lipoxygenase: identification of a novel pathway for regulating allergic inflammation. *J Immunol* 2009;182:5393–5399.
34. Blazquez AB, Berin MC. Gastrointestinal dendritic cells promote Th2 skewing via OX40L. *J Immunol* 2008;180:4441–4450.
35. Drutman SB, Trombetta ES. Dendritic cells continue to capture and present antigens after maturation in vivo. *J Immunol* 2010;185:2140–2146.
36. Moller GM, Overbeek SE, Van Helden-Meeuwsen CG, Van Haarst JM, Prens EP, Mulder PG, Postma DS, Hoogsteden HC. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled corticosteroids. *Clin Exp Allergy* 1996;26:517–524.
37. Hammad H, Charbonnier AS, Duez C, Jacquet A, Stewart GA, Tonnel AB, Pestel J. Th2 polarization by Der p 1-pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood* 2001;98:1135–1141.
38. Huh JC, Strickland DH, Jahnsen FL, Turner DJ, Thomas JA, Napoli S, Tobagus I, Stumbles PA, Sly PD, Holt PG. Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma. *J Exp Med* 2003;198:19–30.
39. Vermaelen KY, Carro-Muino I, Lambrecht BN, Pauwels RA. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *J Exp Med* 2001;193:51–60.
40. Scholer A, Hugues S, Boissonnas A, Fétler L, Amigorena S. Intercellular adhesion molecule-1-dependent stable interactions between T cells and dendritic cells determine CD8+ T cell memory. *Immunity* 2008;28:258–270.
41. Mahnke K, Bhardwaj RS, Luger TA, Schwarz T, Grabbe S. Interaction of murine dendritic cells with collagen up-regulates allostimulatory capacity, surface expression of heat stable antigen, and release of cytokines. *J Leukoc Biol* 1996;60:465–472.
42. Brand U, Bellinghausen I, Enk AH, Jonuleit H, Becker D, Knop J, Saloga J. Influence of extracellular matrix proteins on the development of cultured human dendritic cells. *Eur J Immunol* 1998;28:1673–1680.
43. Suri RM, Austyn JM. Bacterial lipopolysaccharide contamination of commercial collagen preparations may mediate dendritic cell maturation in culture. *J Immunol Methods* 1998;214:149–163.
44. Akdis CA. Allergy and hypersensitivity: mechanisms of allergic disease. *Curr Opin Immunol* 2006;18:718–726.
45. Zhang K, Shan L, Rahman MS, Unruh H, Halayko AJ, Gounni AS. Constitutive and inducible thymic stromal lymphopoietin expression in human airway smooth muscle cells: role in chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L375–L382.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.