Essential Role of Lung Plasmacytoid Dendritic Cells in Preventing Asthmatic Reactions to Harmless Inhaled Antigen

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Abstract

Tolerance is the usual outcome of inhalation of harmless antigen, yet T helper (Th) type 2 cell sensitization to inhaled allergens induced by dendritic cells (DCs) is common in atopic asthma. Here, we show that both myeloid (m) and plasmacytoid (p) DCs take up inhaled antigen in the lung and present it in an immunogenic or tolerogenic form to draining node T cells. Strikingly, depletion of pDCs during inhalation of normally inert antigen led to immunoglobulin E sensitization, airway eosinophilia, goblet cell hyperplasia, and Th2 cell cytokine production, cardinal features of asthma. Furthermore, adoptive transfer of pDCs before sensitization prevented disease in a mouse asthma model. On a functional level, pDCs did not induce T cell division but suppressed the generation of effector T cells induced by mDCs. These studies show that pDCs provide intrinsic protection against inflammatory responses to harmless antigen. Therapies exploiting pDC function might be clinically effective in preventing the development of asthma.

Key words: asthma • plasmacytoid dendritic cells • tolerance • mucosal immunity • regulatory T cell

Introduction

Asthma is an increasingly common disease that remains poorly understood and difficult to manage. Its incidence has doubled in westernized countries in the last two decades and worldwide costs are estimated to exceed those from tuberculosis and HIV/AIDS combined (1), necessitating a way to prevent this disorder. Asthma is a Th2 lymphocyte–mediated inflammatory airway disease characterized by airway eosinophilia, increased mucus production by goblet cells, and structural remodeling of the airway wall. This leads to variable airway obstruction and to bronchial hyperresponsiveness to nonspecific stimuli. In allergic asthma, the presence of high levels of allergen-specific IgE are a reflection of an aberrant Th2 cell immune response to common inhaled environmental allergens such as house dust mite or pollen allergen (2). This process of Th2 cell sensitization to inhaled allergens occurs at a very young age and is influenced by environmental factors such as childhood infections and environmental exposure to microbial compounds (3).

It is currently unknown how exposure to harmless inhaled antigen such as allergen leads to prolonged Th2 cell sensitiza-

tion in individuals with allergy, as respiratory exposure to harmless antigen is a tolerogenic event (4). Recent evidence shows that airway DCs are at the focal control point determining the induction of pulmonary immunity or tolerance (5–8). Airway DCs form a dense network in the lung ideally placed to sample antigens and migrate to draining LNs to stimulate naive T cells (9–12). Airway DCs play a central role not only in initiating specific Th2 cell immune responses leading to experimental asthma (13, 14), but they also restimulate effector cells during ongoing airway inflammation (8, 15–17). Less is known about the tolerogenic capacity of airway DCs. The immune response leading to inhalation tolerance is accompanied by a considerable degree of primary T cell division in draining cervical and mediastinal LNs (MLNs) and therefore it is likely that it also involves antigen presentation by professional APCs (4, 18–21). In support of this theory, DCs obtained from lung draining LNs of tolerized mice were able to induce T cell unresponsiveness ex vivo and transfer tolerance to naive mice, in a process that required active T cell costimulation through either CD86 or ICOS-L (3, 5, 21). Despite the occurrence of inhalational tolerance, some authors have witnessed sensitization to inhaled inert antigen, particularly when signals activating the innate immune system were coadministered (14, 22).

As inhalation of harmless antigen induces sensitization or tolerance in a process controlled by lung DCs, we hypoth-

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esized that a more detailed study on the functional outcome of antigen presentation by subsets of DCs in the lung might provide insight into the decision governing tolerance or immunity. We found that particular subsets of DCs were able to take up and transport antigen in the lung. Whereas myeloid DCs (mDCs) were important for generating T cell division and priming, plasmacytoid DCs (pDCs) suppressed T cell effector generation. Strikingly, in the absence of pDCs, exposure to harmless antigen led to Th2 cell sensitization and to features of asthma.

Materials and Methods

Mice. 6–8-wk-old BALB/c mice were purchased from Harlan. OVA-TCR transgenic mice (DO11.10) on a BALB/c background were bred at the Erasmus Medical Center. All experiments were performed according to institutional guidelines of the animal ethics committee at Erasmus Medical Center.

Isolation of Bronchoalveolar, Lung, and LN Cells. After anesthesia with 2.5% avertin, mice were bled and bronchoalveolar lavage (BAL) was performed using 3 × 1 ml warm PBS containing 0.1 mM EDTA through a canula placed in the trachea (10). To obtain single lung cell suspensions, lungs were perfused with 20 ml PBS through the right ventricle, minced using iridectomy scissors, and digested with collagenase III and DNase I, as described previously (11). For obtaining single cell suspensions from LNs, MLNs were excised, minced, and digested as described above. After blocking the reaction with excess EDTA, cells (>95% viability) were washed and stained for flow cytometry.

Flow Cytometry and Sorting. All staining reactions were performed at 4°C. First, cells were incubated with 2.4G2 Fc receptor Ab to reduce nonspecific binding. Dead cells and debris were excluded using propidium iodide. To detect DCs, single cells were stained with APC-labeled anti-CD11c (HL3) and FITC-labeled anti–Gr-1 (RB6-8C5) Abs. The phenotype of $CD11c^{int}$ $Gr-1^+$ (pDCs) and $CD11c^{hi}$ $Gr-1^-$ (mDCs) cells was determined by using PE-labeled anti-B220 (RA3-6B2), CD8α (Ly-2), CD19 (ID3), CD4 (RM4-5), CD40 (3/23), CD80 (16-10A1), CD86 (GL-1), CD54 (3E2), CD62L (MEL14), CD24 (M1/69), MHC II (2G9), CD45 RB (16A), and pan NK (DX5) Abs (all from BD Biosciences), and anti–PD-L1 (MIH5) and anti–PD-L2 (Ty25; eBioscience). In experiments where FITC-OVA uptake was studied, DCs were detected by PE-labeled anti–Gr-1 and APC-labeled anti-CD11c Abs.

In some experiments, total lung and LN cells were sorted based on CD11c-APC and Gr-1-FITC staining using a FACS-DiVa® flow cytometer (BD Biosciences). Cytospins of the different sorted lung cell populations were stained with May-Grunwald Giemsa.

Confocal Microscopy. Confocal analysis was performed on 6-μm cryostat sections of perfused lungs stained with anti–Gr-1 FITC and anti-B220-PE to detect pDCs. Sections were analyzed on a confocal laser microscope (LSM-510; Carl Zeiss MicroImaging, Inc.). To detect intracellular uptake of FITC-OVA, cytospins of $CD11c^{int}$ $Gr-1^+$ cells sorted from MLNs taken 36 h after FITC-OVA administration were stained with anti-B220-PE, and FITC signal was observed using confocal laser microscopy.

Functional Activity of DC Subsets. To detect the antigen uptake capacity of DC subsets, mice received an intratracheal (i.t.) injection of 800 μg FITC-OVA (screened for low LPS content; Molecular Probes) in a volume of 80 μl PBS. Draining MLNs were excised 36 h later and analyzed by flow cytometry. To study

antigen-presenting capacity of DC subsets ex vivo, mice first received an i.t. injection of 800 μg OVA (LPS content, <20 pg/mg OVA; Seikagaku). Next, $CD11c^{int}$ $Gr-1^+$ $B220^+$ (pDCs) and $CD11c^+$ $Gr-1^-$ $B220^-$ cells (mDCs) were sorted from the MLNs 36 h later and cocultured with purified $CD4^+$ (purity, >95% using magnetic bead purification; Miltenyi Biotec) naive carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled DO11.10 TCR transgenic T cells at a 1:10 ratio. The labeling of T cells with CFSE was essentially as described previously (10). The stimulatory capacities of lung DC subsets were compared with those of in vitro OVA-pulsed pDCs or mDCs grown from BM cultures in the presence of Flt3L or GM-CSF, respectively. Generation of BM DCs was essentially as described previously (23). 4 d later, proliferation of $CD4^+$ $KJ1-26^+$ T cells was determined by flow cytometry.

As IFN-α production by pDCs is a defining characteristic of these cells, we cultured sorted $CD11c^{int}$ $Gr-1^+$ and $CD11c^{hi}$ $Gr-1^-$ DCs in the presence or absence of 2 μM CpG-oligodeoxynucleotides (5′-TGACTGTG-AACGTTCCGAGATGA-3′; Sigma-Aldrich). Supernatants were collected 24 h later and analyzed by ELISA for the presence of IFN-α (PBL Biomedical Laboratory, Inc.).

Immune Response to Inhaled OVA in the Presence or Absence of pDCs. Next, we studied the immune response to harmless inhaled OVA in mice with or without pDCs. For depletion of pDCs, 250 μg/ml depleting anti–Gr-1 or control isotype Abs were given on 4 consecutive days (24), starting 1 d before i.t. priming with 800 μg LPS^{low} OVA (LPS contamination of 2.9 ng/mg protein; Worthington) or, to avoid confounding effects of LPS, with 800 μg LPS-free OVA (LPS, <20 pg/mg protein; Seikagaku). In separate experiments, mice received four i.p. injections of pDC-selective depleting 120G8 Ab (50 μl ascites/day; provided by C. Asselin-Paturel, Schering-Plough, Dardilly, France; reference 25). On day 4, the depletion of $CD11c^{int}$ $Gr-1^+$ cells was evaluated on total lung cells and MLNs. Next, we addressed whether mice were sensitized to inhaled OVA by rechallenging with OVA aerosol. After a wash out period of 20 d, mice received 3 OVA aerosols (10 mg/ml in PBS, generated using a jet nebulizer) of 30 min on 3 consecutive days. 24 h later, BAL fluid was taken and analyzed by flow cytometry as described previously (26). In brief, eosinophils were characterized as $CCR3^+$ SSC^{hi} cells, neutrophils as $CCR3^-$ SSC^{hi} cells, lymphocytes as $B220^+/CD3^+$ FSC^{lo} cells, and macrophages as large autofluorescent cells. To measure cytokine levels, MLN cells were plated in round-bottom 96-well plates (10^6 cells/ml) and restimulated with 10 μg/ml OVA for 4 d. Production of IL-4, IL-5, IL-10, IL-13, and IFN-γ was assayed on supernatants by ELISA (BD Biosciences) as described previously (12). Levels of OVA-specific IgE were measured by ELISA as described previously (13). In addition, lungs were resected and embedded in HistoWax (Intertiles). 4-mm sections were stained with May-Grunwald Giemsa.

To study primary T cell activation in the lung in the presence or absence of pDCs, mice received an i.v. injection of $10 × 10^6$ CFSE-labeled DO11.10 T cells day −1 before injection of 0, 8, 80, and 800 μg LPS-free OVA (day 0). From day −1 to 2, the mice received an i.p. injection of anti–Gr-1 Abs or control isotype Abs (250 μg/day). T cell responses were analyzed in the draining MLNs at day 4 by observing CFSE division profiles of live $KJ1-26^+$ $CD4^+$ T cells. Cytokine levels were measured as described above.

Induction of Regulatory T (T reg) Cells by Lung pDCs. To detect induction of T reg cells, we used a method recently described by Martin et al. (27). First, 800 μg LPS-free OVA was in-

jected i.t. and $CD11c^{int}$ $Gr-1^+$ $B220^+$ cells were sorted from the MLNs 36 h later. Sorted DCs were cultured with naive DO11.10 T cells for 3 d at a 1:10 ratio. In the second phase, pDC-stimulated T cells were replated and cultured at 5×10^5 cells per well in the presence of 1 ng/ml mouse IL-2 (R&D Systems) for an additional 7 d without OVA. The T reg cell assay was performed by culturing freshly purified DO11.10 $CD4^+$ T cells (10^5 per well) with irradiated BALB/c splenocytes (10^5 per well) in 96-well plates with 10 μg/ml $OVA_{323-339}$ peptide in the presence or absence of pDC-stimulated T cells (10^5 per well). Cell proliferation was assessed after 48 h by [^{3}H]thymidine (1 μCi/well) uptake in a 16-h pulse.

Adoptive Transfer of pDCs into the Lung. $CD11c^+$ $CD11b^-$ $B220^+$ pDCs were sorted from Flt3L-cultured BM as described previously using a FACSDiVa® flow cytometer (23, 28). 100 μg/ml OVA was added during the last 24 h of culture. 4×10^5 OVA-pulsed or -unpulsed pDCs were injected i.v. three times on alternate days. 1 wk later, mice received an i.p. injection of OVA-alum (10 μg OVA adsorbed to 1 mg aluminiumhydroxide;

Sigma-Aldrich; reference 15). Mice were rechallenged three times with OVA aerosols 10 d later, as described above. 24 h after the last aerosol exposure, BAL fluid was performed and cell differential counts were analyzed by flow cytometry (26).

Statistical Analysis. For all experiments, the difference between groups was calculated using the Mann-Whitney U test for unpaired data. Differences were considered significant when $P < 0.05$.

Online Supplemental Material. Data showing the phenotype of DC subsets and IFN-α production by DCs from the lung (Fig. S1) and the induction of tolerance by a single i.t. injection of OVA (Fig. S2) are available at: http://www.jem.org/cgi/content/full/jem.20040035/DC1.

Results

Different Subtypes of DCs Are Present in the Lung. In humans and mice, there are two major subsets of DCs, called mDCs and pDCs. pDCs are known to be present in he-
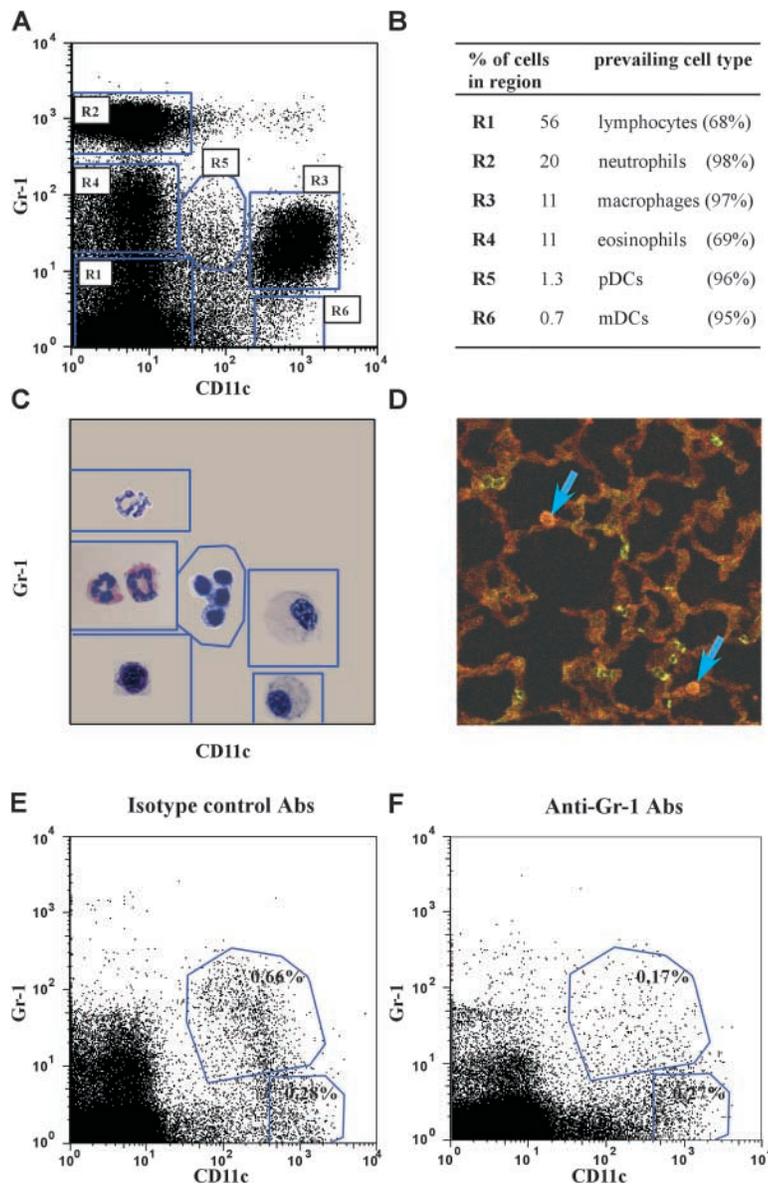

Figure 1. Identification of pDCs in the lung. (A and B) After digestion of the lungs, cells were stained for Gr-1 and CD11c expression, gated on live cells, and analyzed by flow cytometry. At least six different leukocyte populations were identified (regions R1–R6). (C) All leukocyte populations were sorted on Gr-1 and CD11c expression and stained with May-Grunwald Giemsa. Cytospins showed typical morphology of lymphocytes (R1), neutrophils (R2), macrophages (R3), eosinophils (R4), pDCs (R5), and mDCs (R6). Magnification, 100. (D) Confocal microscopy of frozen lung section stained with anti–Gr-1 FITC (green) and anti–B220-PE (red) Abs. Double positive colocalization signal appears orange (blue arrows). (E) Digested MLNs of naive mice were stained for Gr-1 and CD11c expression and gated on live cells. $CD11c^{int}$ $Gr-1^+$ pDCs and $CD11c^{hi}$ $Gr-1^-$ mDCs are shown. (F) MLNs of mice receiving anti–Gr-1 Abs for 4 d were digested and stained as described above. Depletion of $CD11c^{int}$ $Gr-1^+$ pDCs but not $CD11c^{hi}$ $Gr-1^-$ mDCs is shown.

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matopoietic organs (29, 30), blood (31), and lymphoid organs (32–35), and play a critical role in mounting the protective immune response to virus infection by producing large amounts of IFN-α (31, 32, 34). Although lung mDCs have been described in detail, it is not known if lung also contains pDCs or what the function of these cells might be. To analyze the subsets and numbers of DCs present in the lung of naive mice, whole lungs were digested and stained for Gr-1 and CD11c, markers known to distinguish between mDCs (CD11c^{hi} Gr-1$^-$) and pDCs (CD11c^{int} Gr-1$^+$) in the spleen and LNs (35). Several populations of lung leukocytes could be discriminated by their expression of Gr-1 and CD11c (Fig. 1, A and B). When sorted, the different populations showed typical morphology of lymphocytes, neutrophils, macrophages, eosinophils, pDCs, and mDCs (Fig. 1 C). At least 50% of the pDCs gated in region R5 were B220$^+$ (27, 35) and expressed CD45RB$^+$ (34), yet none expressed the classical B cell markers or the "lymphoid" marker CD8α, expressed on various subsets of spleen DCs (reference 36; Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20040035/DC1). Furthermore, pDCs had a more immature phenotype than mDCs as assessed by their lower expression of costimulatory molecules and MHC II. On the contrary, the inhibi-

tory B7 family costimulatory molecule PD-L1 was highly expressed on pDCs compared with mDCs (mean fluorescence intensity: 409.5 $\pm$ 42.5 and 328.5 $\pm$ 44.5, respectively; Fig. S1 B).

Confocal microscopy of lung tissues of naive mice confirmed that B220$^+$ Gr-1$^+$ pDCs are a major subset of DCs that are mainly located in the lung interstitium (Fig. 1 D). Only CD11c^{int} Gr-1$^+$ cells were able to produce IFN-α in response to CpG-oligodeoxynucleotides (Fig. S1 C), a defining functional characteristic of pDCs (24, 31). As a positive control, BM-derived pDCs grown in Flt3L produced very similar levels of IFN-α, whereas lung mDCs or BM-derived mDCs did not. As expected, CD11c^{int} Gr-1$^+$ pDCs were also found in the MLNs of naive mice as described previously by others (reference 24; Fig. 1 E).

Lung pDCs Prevent Sensitization to Inhaled Harmless Antigen. Next, we questioned if pDCs influence the decision between tolerance or immunity to inert antigens in the lung (5). In our protocols using commercially available OVA screened to contain no or very low levels of LPS endotoxin, a single i.t. injection of OVA did not lead to sensitization or to Th2 cell–associated eosinophilic airway inflammation upon repeated challenge with OVA aerosol, and even lead to suppression of airway inflammation in a
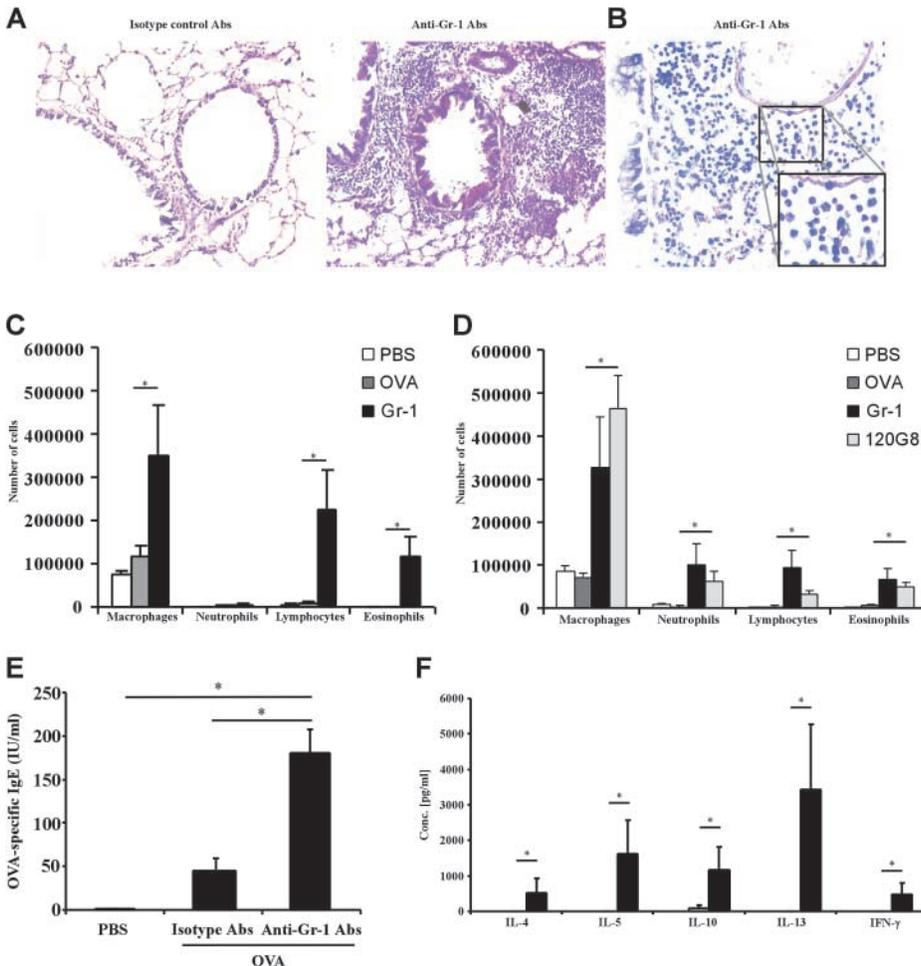

Figure 2. Role of pDCs in asthma. (A) On day 0, mice received an i.t. injection of LPS^{low} OVA. From days -1 to 2, mice were injected i.p. with anti–Gr-1 Abs or isotype control Abs. 20 d later, mice were exposed to three OVA aerosols. Lungs of mice depleted or not of pDCs were stained with periodic acid-Schiff. (B) Lungs were also stained with May-Grunwald Giemsa to detect eosinophils. (C) BAL fluid of mice injected with anti-Gr1 Abs (solid bars) or with isotype control Abs (shaded bars), and of naive mice (open bars) was analyzed by flow cytometry. Data are shown as mean $\pm$ SEM. *, P < 0.05. (D) Experiments were repeated using LPS-free OVA and the depleting Ab 120G8, given at the same time points as for Gr-1. (E) Sera from mice depleted or not from pDCs were analyzed for the presence of OVA-specific IgE. Results are shown as mean $\pm$ SEM. *, P < 0.05. (F) MLN cells collected from mice injected with anti-Gr1 Abs (solid bars) or with isotype control Abs (shaded bars), and of naive mice (open bars) were plated and restimulated in vitro for 4 d with OVA. Supernatants were assayed for IL-4, IL-5, IL-10, IL-13, and IFN-γ production. Data are shown as mean $\pm$ SEM. *, P < 0.05.

classical alum-adjuvant–driven animal model of asthma, a defining characteristic of true immunological tolerance (Fig. 2 A and Fig. S2, A and B, available at http://www.jem.org/cgi/content/full/jem.20040035/DC1). To specifically address the role of pDCs in this process, pDCs were depleted using anti–Gr-1 Abs. Flow cytometric staining on draining MLNs (Fig. 1 F) confirmed that this Ab depleted pDCs but not mDCs as described previously by others (24). However, in the lung, pDCs were not as strongly depleted as in the MLNs (unpublished data). pDCs were depleted during a first exposure to OVA antigen given by i.t. injection. When challenged with OVA aerosols 3 wk later, control mice, as expected (14), did not develop features of asthma (Fig. 2 A). In contrast, pDC-depleted mice developed cardinal features of asthma as shown by the eosinophilic inflammation around bronchi and blood vessels, the occurrence of goblet cell hyperplasia (Fig. 2, A and B), eosinophilia in the bronchoalveolar com-

partment (Fig. 2 C), and the presence of OVA-specific IgE in the serum (Fig. 2 E). These characteristics of asthma in pDC-depleted mice were associated with a substantial increase in Th2 cell cytokine production in MLNs (Fig. 2 F). These experiments were performed with OVA containing 2.9 ng LPS/mg OVA protein, potentially confounding our study (14). Moreover, the Gr-1 Ab can also deplete neutrophils or other Gr-1$^+$ cells. Therefore, the experiments were also performed with LPS-free OVA using a pDC-selective depleting Ab 120G8. Under these conditions, exposure to LPS-free OVA also led to sensitization to inhaled OVA (Fig. 2 D). These observations are strong evidence that lung pDCs suppress sensitization to otherwise harmless tolerogenic protein antigens.

Functional Activities of Lung pDCs. Next, we examined how lung pDCs might influence the immune response to inhaled antigens. Up to now, it is uncertain whether pDCs can take up whole antigens (besides viruses) for processing
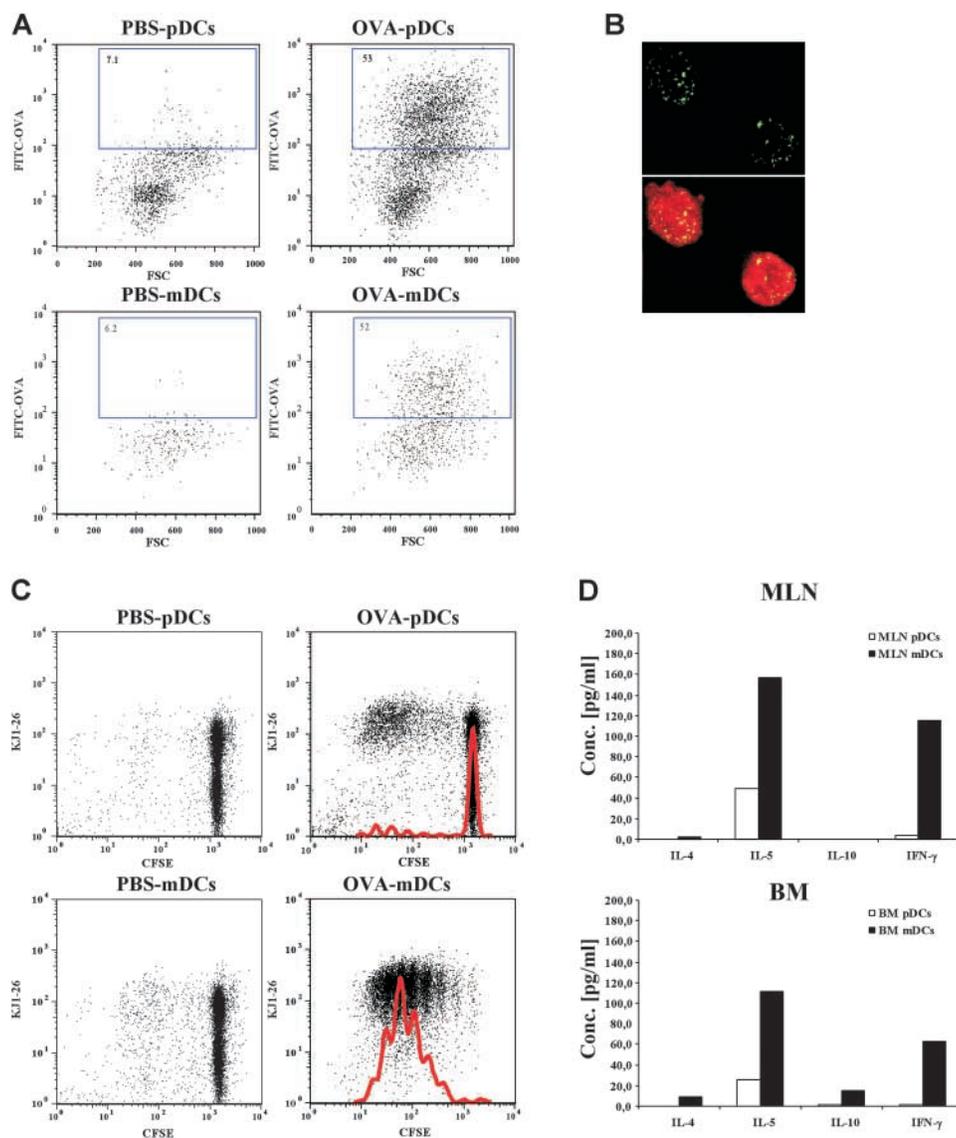

Figure 3. pDCs transport OVA from the lung to the draining LNs but fail to prime naive T cells in vitro. (A) Mice were injected i.t. with FITC-OVA. 2 d later, MLNs were analyzed for the presence of OVA-loaded pDCs (Gr-1$^+$ CD11c^{int}) and mDCs (Gr-1$^-$ CD11c^{hi}). (B) Confocal analysis of FITC-OVA uptake by pDCs. Mice were injected i.t. with FITC-OVA. 2 d later, cytospins of CD11c^{int} Gr-1$^+$ FITC-OVA$^+$ cells sorted from MLNs were prepared and stained with anti-B220 PE Abs. FITC$^+$ cells (green, top) are also positive for B220 (red, bottom) (C) Mice were injected i.t. with unlabeled OVA. 2 d later, pDCs (gated for Gr-1$^+$ CD11c^{int}) and mDCs (gated for Gr-1$^-$ CD11c^{hi}) were sorted from MLNs and cocultured with purified CFSE-labeled CD4$^+$ T cells from DO11.10 mice. 4 d later, proliferation of OVA-specific T cells was determined. (D) Cytokine production by T cells in vitro stimulated with pDCs. OVA-pulsed pDCs and mDCs were sorted from MLNs (top) or from the BM (bottom), and cocultured for 4 d with purified CD4$^+$ T cells from DO11.10 mice. Cytokines were assayed in the supernatants.

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and presentation. Therefore, FITC-labeled OVA was given i.t. (11). pDCs as well as mDCs were able to take up FITC-OVA locally in the lung (unpublished data). Strikingly, after migration to the draining MLNs 36 h later, only mDCs and pDCs were FITC-OVA+. The number of FITC-OVA+ pDCs in the MLNs was higher than FITC-OVA+ mDCs (Fig. 3 A). At this time point, >50% of the pDCs and mDCs present in the MLNs were positive for FITC-OVA. Moreover, as shown by confocal microscopy, FITC-OVA was present inside vacuoles of B220+ pDCs (Fig. 3 B). As both FITC-OVA–loaded pDCs and mDCs were present in MLNs, their capacity to stimulate naive TCR-transgenic OVA-specific T cells ex vivo was compared. Only mDCs loaded with OVA in vivo were able to induce a strong proliferation (78.5% OVA-specific T cells had divided in response to mDCs vs. only 2.37% with pDCs; Fig. 3 C). Moreover, OVA-loaded mDCs obtained from MLNs or derived from the BM induced production of both Th1 and Th2 cell cytokines by T cells, whereas pDCs from the lung and BM did not produce cytokine levels above the level of unstimulated T cells (Fig. 3 D).

pDCs Suppress the Generation of Effector Cells by mDCs In Vivo.   Next, we examined the contribution of pDCs to T cell division induced by various doses of LPS-free OVA inhalation by depleting with anti–Gr-1 Abs (37). As the direct examination of naive T cell activation is impossible due to the low precursor frequency, we adoptively transferred naive OVA-specific T cells from DO11.10 TCR transgenic mice. As shown previously (12), without OVA inhalation, DO11.10 T cells did not divide, whereas after inhalation of increasing doses of OVA, vigorous proliferation occurred 4 d later in OVA-specific CD4+ T cells of the MLNs (Fig. 4 A). T cell proliferation was of similar magnitude in the presence or absence of pDCs regardless of the dose of OVA used (Fig. 4 A), consistent with the in vitro observation that pDCs by themselves were unable to induce T cell proliferation. Although no apparent difference was detected in terms of T cell proliferation between mice depleted of pDCs or not, there was a significant increase in the amount of effector cytokines (IL-5, IL-10, IL-13, and IFN-γ) produced by proliferating T cells taken from immunized pDC-depleted animals (Fig. 4 B, 800-μg dose). This suggests that lung pDCs suppress the generation of effector function in naive T cells, which proliferate in response to Ag presentation by mDCs, and provide a mechanism for the occurrence of sensitization in the absence of pDCs.

pDCs Induce the Generation of Suppressive T Cells In Vitro.   Next, we investigated whether pDCs could be endowed with a tolerogenic potential by inducing T cell unresponsiveness and the differentiation of T reg cells. T cells that were first stimulated in vitro with pDCs obtained
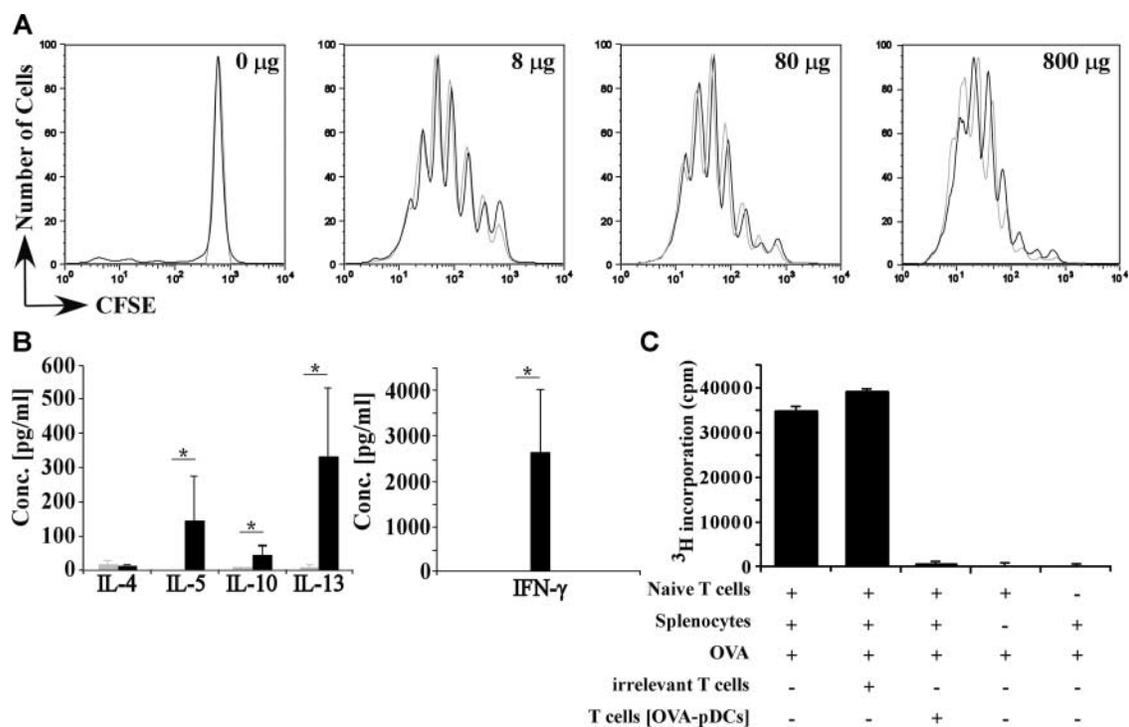

Figure 4.   pDCs down–regulate the immune response in vivo without affecting T cell division. On day −1, mice were injected i.v. with CFSE-labeled T cells from DO11.10 mice. On day 0, they received an i.t. injection of different doses of LPS-free OVA (0, 8, 80, and 800 μg). (A) From days −1 to 2, mice were injected i.p. with anti–Gr-1 Abs (gray line) or isotype control Abs (black line), and proliferation of CFSE-labeled OVA-specific T cells was determined at day 4 in MLNs. (B) MLN cells were plated for 4 d and supernatants were assayed for cytokine production from mice injected with anti-Gr1 Abs (solid bars) or isotype control Abs (shaded bars). Data are mean ± SEM. (C) T reg cell assay. Proliferation of OVA-specific T cells after a 2-d coculture with syngeneic splenocytes and OVA peptide in the presence of T cells previously stimulated with OVA-pulsed pDCs (T cells [OVA-pDCs]) or irrelevant BALB/c T cells. Cell proliferation was assessed by [³H]thymidine (1 μCi/well) uptake in a 16-h pulse. Data are mean ± SEM for triplicate cultures.

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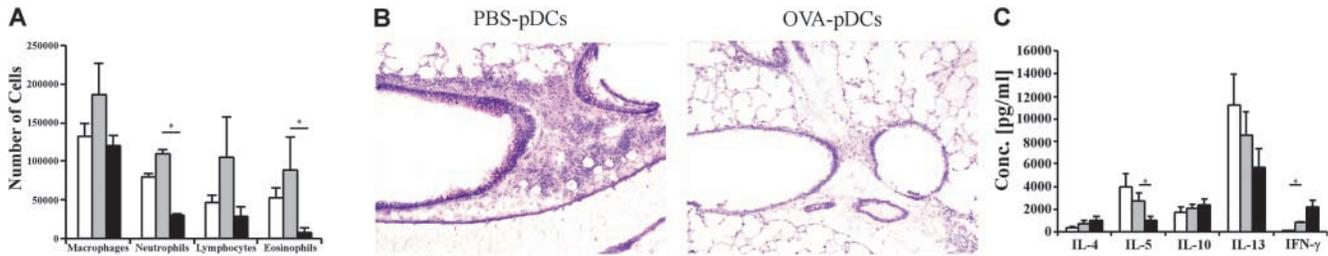
Figure 5. Adoptive transfer of pDCs protects against asthma development. pDCs were sorted from Flt3L-cultured BM, pulsed (solid bars) or not (shaded bars) with OVA, and injected i.v. three times on alternate days. As a control, some mice were not injected with pDCs (open bars). 1 wk later, all mice were immunized with OVA-alum i.p. and rechallenged three times with OVA aerosols 10 d later. (A) BAL fluid was analyzed by flow cytometry as in Fig 2. Data are mean ± SEM. *, P < 0.05. (B) Lungs from mice injected with OVA-pulsed or –unpulsed pDCs were stained with May-Grunwald Giemsa. (C) MLN cells were plated for 4 d in the presence of 10 μg/ml OVA. Supernatants were assayed for the presence of cytokines. Data are mean ± SEM. *, P < 0.05.

from the MLNs of OVA-exposed mice strongly reduced the proliferation of freshly purified naive T cells from DO11.10 mice induced by OVA peptide–pulsed splenocyte APCs. This was not due to dilution or to competition for IL-2, as polyclonal wild-type BALB/c T cells added to naive DO11.10 T cells and splenocytes in the same proportion did not lead to reduced proliferation (Fig. 4 C). In conclusion, our data suggest that pDCs induced the differentiation of T reg cells capable of suppressing antigen-specific T cell proliferation.

Adoptive Transfer of pDCs Prevents Development of Asthma. Together, these data show that lung pDCs provide an intrinsic mechanism to suppress inflammation in response to inhalation of harmless antigen, and therefore might provide a mechanism to prevent the occurrence of sensitization. To more directly demonstrate this intrinsic tolerogenic capacity of pDCs, BM-derived OVA-pulsed pDCs (29) were injected three times i.v. before subjecting mice to a fully immunogenic asthma protocol consisting of sensitization by i.p. injection of OVA in alum, followed by OVA aerosol challenge (38). As expected, mice receiving no pDCs or unpulsed pDCs uniformly developed eosinophilic airway inflammation and goblet cell hyperplasia (Fig. 5, A and B). In contrast, administration of OVA-pulsed pDCs before OVA-alum sensitization was able to strongly inhibit the aerosol-induced BAL eosinophilia (Fig. 5 A), airway inflammation (Fig. 5 B), and the production of Th2 cell cytokines by MLN T cells (Fig. 5 C). Although we did not use lung pDCs because of technical difficulty in obtaining sufficient cells, these data support the idea that the intrinsic tolerogenic capacity of pDCs can be exploited to prevent the onset of asthma.

Discussion

By showing the presence and antigen uptake of pDCs in the lung, by demonstrating direct priming to antigen in the absence of pDCs, and by demonstrating transfer of the tolerizing effect through injection of pDCs, we believe that pDCs have an essential immunoregulatory role in the lung that protects against development of airway inflammation to harmless antigens.

To our knowledge, this is the first description of pDCs in the lung of mice. These cells demonstrate intermediate expression of CD11c, express Gr-1, B220, and CD45RB, and they produce large amounts of IFN-α after stimulation with CpG motifs. These cells take up inhaled FITC-OVA protein and migrate to the MLNs draining the lung carrying their fluorescent cargo inside vacuoles. We believe that these cells are most likely related to the pDCs also described in the draining LNs and spleen of mice (35), and are distinct from the CD45RB+ DCs recently described by Wakkach et al. (39) by their expression of Gr-1. We are currently studying if these CD45RB+ Gr1− DCs are also present in the lung.

In the absence of pDCs during primary OVA exposure to the lung, sensitization was induced, which led to features of Th2 cell–associated asthma after inhaling harmless antigen. This is a remarkable observation, as primary exposure to inhaled OVA in our system usually leads to inhalational tolerance, even when mice are immunized with OVA in alum (Fig. S2; references 18–21). For our studies in which we depleted pDCs, we have used Gr-1 Abs that also deplete neutrophils (24). We do not believe that absence of neutrophils had a predominant effect on T cell priming, as Gr-1+ neutrophils did not carry Ag to the node. However, to more directly prove a role for pDCs as tolerogenic cells, we used another, more selective pDC-depleting Ab, 120G8, and found identical results, arguing against a predominant role for the neutrophil (25). Moreover, we performed adoptive transfer experiments before a fully immunogenic asthma protocol. Adoptive transfer of OVA–pulsed BM-derived pDCs (BM-pDCs) before induction of asthma completely inhibited airway inflammation. This response required the presence of OVA, as unpulsed pDCs did not suppress airway inflammation. Remarkably, under the same conditions, BM-mDCs did not exert this effect and even strongly enhanced the cardinal features of asthma (unpublished data and 13).

Next, we performed experiments to elucidate the mechanism by which absence of pDCs might lead to sensitization. It is known that inhalational tolerance to harmless antigen is accompanied by a significant amount of primary T cell activation in the draining nodes, and therefore it was

suggested that depletion of pDCs might influence T cell division. To our surprise, however, we noticed no difference in the primary division of naive T cells when the outcome was either immunity (i.e., in the case of depletion of pDCs) or tolerance (treatment with control Abs), illustrating that the strength of primary T cell division does not predict the functional outcome of a T cell response. Moreover, removing pDCs during OVA inhalation resulted in higher levels of effector Th2 (IL-5, IL-10, IL-13) and Th1 cell cytokines (IFN-γ) being produced by T cells in the draining MLNs. Indirectly, these data show that the mDC subset is the predominant APC responsible for inducing naive T cell proliferation in the draining nodes. The uncoupling of T cell proliferation from generation of effector potential and from differentiation into memory cells is emerging as a new concept in T cell biology (4, 40). Lanzavecchia and Sallusto (41) recently proposed that T cell stimulation of insufficient strength or duration leads to proliferation of T cells that are "unfit" to respond to T cell survival signals and do not produce effector cytokines. The fact that in our experiments T cell proliferation was accompanied by proinflammatory effector T cell generation only when pDCs were depleted, suggests that pDCs are inefficient APCs that compete with immunogenic mDCs for antigen presentation, thus effectively reducing the overall strength or duration of T cell stimulation. From our and previous studies there is evidence that pDCs are weak APCs. Only when pDCs were pulsed with various doses of OVA peptide in vitro, could either Th1 or Th2 cell responses be induced (23). The fact that lung mDCs and pDCs take up fluorescent OVA does not imply that both cells provide an identical TCR ligand density, as there might be significant differences in antigen processing and presentation efficiency (32). Alternatively, pDCs in the lung and MLNs are immature, expressing low levels of MHC II and costimulatory molecules, yet high levels of the inhibitory B7 family member PD-L1 molecule, known to suppress T cell activation through interaction with PD-1 (42). This profile of high inhibitory and low costimulatory molecule expression together with the poor T cell stimulatory capacity ex vivo, point out to the "tolerogenic" potential of pDCs in the lung. It remains to be demonstrated whether the depletion of pDCs during priming would also lead to longer survival of responding T cells as would be predicted from the "T cell fitness" model (40, 41). Alternative explanations could be that immature lung pDCs are important for generating T reg cells after exposure of harmless antigen, as proposed in vitro by others (27, 29, 39, 43) and also shown indirectly ex vivo here (Fig. 4 C). In this scenario, in the absence of pDCs, T reg cells would no longer be induced from naive T cells or expanded from a pool of naturally occurring OVA–responsive CD4$^+$ CD25$^+$ regulatory cells (44, 45). The exact phenotype and function of these T reg cells induced by lung pDCs remains to be determined.

Concomitant exposure of harmless antigen with signals that activate innate immune responses such as viral infection, bacterial LPS, or GM-CSF, lead to a break in inhala-

tional tolerance presumably through activation of mDCs (4, 7, 14, 46). Supporting this idea, injection of mature mDCs but not pDCs indeed leads to Th2 cell sensitization in the lung (unpublished data and 13). We observed induction of Th2 cell sensitization in response to LPS-free OVA inhalation, which was shown by us (Fig. S2) and by others (47) to lead to tolerance and did not induce any form of mDC activation (unpublished data). Therefore, we believe that modulation of the intrinsic tolerogenic function of pDCs represents a second mechanism by which "adjuvant factors" lead to Th2 cell sensitization to harmless antigens. In this context, there is a known association between respiratory viral infections such as respiratory syncytial virus at a young age and the development of asthma (48). As viruses strongly interact with pDCs, we propose that inhibition of the normal tolerogenic activities of lung pDCs by viral infection could be the mechanism behind this association. Viral infections induce maturation of pDCs in vivo and this might explain the breaking of inhalational tolerance (37). Some "adjuvants" might also alter the ratio between immunogenic mDCs and pDCs in the lung and thus influence the decision between immunity and tolerance. This was nicely shown in the case of adenoviral transfection of the lung with GM-CSF, a predominant growth factor for mDCs but not pDCs, which led to sensitization (46). It will be interesting to study if increasing pDCs would have the opposite effect. It is striking that administration of CpG motifs to the lung leads to strong accumulation of lung pDCs (unpublished data) and to protection from asthma through unclear mechanisms not mediated by Th1 cell cytokines (49).

The discovery that pDCs influence the generation of T cell effector responses has extensive implications for our understanding of immunoregulation in a wide range of T cell–mediated diseases. Inhibition of the tolerogenic capacity of pDCs could thus offer an explanation why some viral infections lead to a break in peripheral self-tolerance in a number of autoimmune diseases such as diabetes (50, 51). In light of our findings, it will be very interesting to see whether the function of lung pDCs in humans with allergy or asthma would also be reduced, thus favoring Th2 cell sensitization to inhaled antigen (52). One recent report showed that the number of circulating pDCs in children with atopic asthma was reduced compared with healthy controls (53).

In conclusion, we have identified a role for endogenous pDCs in the protection against asthmatic reactions to harmless antigens by demonstrating that asthma develops in the absence of pDCs. Therefore, aberrant function of pDCs might contribute to allergic sensitization. Therapies targeted at amplifying the intrinsic tolerogenic capacity of lung pDCs may limit the development of asthma.

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