

Lung dendritic cells induce T_H17 cells that produce T_H2 cytokines, express GATA-3, and promote airway inflammation

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Background: Dendritic cells (DCs) are crucial to shape the adaptive immune response. Extensive *in vitro* manipulation reprograms T_H2 and T_H17 cell lines into T_H1 cells, leading to the concept of CD4⁺ T_H cell subset plasticity. The conversion of memory T_H17 cells into T_H2 cells or vice versa remains to be clarified.

Objective: We examined the localization of T_H17/T_H2 cells *in vivo*, their cellular origin (T_H2 vs T_H17), and the underlying mechanisms that drive the generation of these double T_H producers.

Methods: Antigen-loaded bone marrow–derived DCs (ovalbumin-DCs) were repeatedly administered locally (intratracheally) or systemically (intravenously) to naive mice to elicit chronic airway inflammation. Inflamed lungs and mediastinal lymph nodes were examined for the presence of IL-17⁺IL-13⁺IL-4⁺CD4⁺ T cells that coexpressed retinoic acid receptor–related orphan receptor γ t and GATA-3 (T_H17/T_H2). **Results:** We show that repetitive administration of inflammatory ovalbumin-DCs, locally or systemically, promoted the development of antigen-specific T_H17/T_H2 cells in lungs and mediastinal lymph nodes. Immunized mice had IgE-independent and steroid-resistant airway inflammation with a mixed neutrophil and eosinophil infiltration of the bronchoalveolar lavage fluid. Airway inflammatory signal regulatory protein α –positive DCs reprogrammed *in vitro*–generated T_H17 but not T_H2 cells, as well as lung effector T_H cells, into T_H17/T_H2 cells.

Conclusion: We demonstrate the existence of T_H17/T_H2 cells that express GATA-3 in inflamed tissues and their T_H17 origin. We further propose that repeated immunization with inflammatory DCs prevails on the route of DC administration to drive T_H17/T_H2-associated chronic lung inflammation. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: T_H17, T_H2, dendritic cells, airway inflammation, signal regulatory protein α

Abbreviations used

APC: Antigen-presenting cell
BMDc: Bone marrow–derived dendritic cell
DC: Dendritic cell
mLN: Mediastinal lymph node
OVA: Ovalbumin
ROR: Retinoic acid receptor–related orphan receptor
SIRP α : Signal regulatory protein α

Different CD4⁺ T_H cells provide hosts with a variety of specific immune responses against infectious pathogens. On the basis of their function and cytokine profile, terminally differentiated CD4⁺ T_H cells are classified into distinct lineages, with the T_H1–T_H2–T_H17 paradigm dominating the field. T_H17, T_H2, and T_H1 cells confer protection against fungi, extracellular, and intracellular microbes, respectively.¹ The concept of functional plasticity of CD4⁺ T_H cell subsets has recently emerged.^{2,3} Regulatory T cells, the primary function of which is to ensure homeostasis within the immune system, can convert into aggressive T_H17, T_H1, or T_H2 cells.^{4,6} T_H17 cells can be reprogrammed into T_H1 cells but not vice versa, whereas recent reports have demonstrated a plasticity of T_H2 cells into T_H1 or T_H17 cells.^{7,8} Although the physiological consequences of this flexibility remain to be determined, it is interpreted as a means of orchestrating adaptive immune responses to a large quantity and vast array of different microbes. Because CD4⁺ T_H subsets mediate immune pathology in addition to their beneficial roles for the hosts, the acquisition of the capacity to produce new cytokines might as well be viewed as a mechanism to regulate immunopathology. By producing IL-10, T_H1 cells dampen the inflammatory response and minimize tissue injury.⁹ In contrast, when converted to T_H1 cells, T_H17 cells enhance immune response and aggravate immune-mediated disease.^{6,10}

T_H2 cells and their cytokines, IL-4, IL-5, and IL-13, are univocally implicated in allergic disorders characterized by eosinophil infiltrates, mucus secretion, and airway remodeling.¹¹ Although the functions of T_H17 cells in allergic airway inflammation have led to conflicting results in mice,^{11,12} a growing list of evidence supports the implication of T_H17 cells in IgE-independent, steroid-resistant neutrophilic asthma *in vivo* in mice and in human subjects.^{13–19}

Airway epithelial cells and dendritic cells (DCs) are primarily involved in the initiation of T_H2 responses through a direct interaction with inhaled antigens. Activated DCs mature and migrate to the mediastinal lymph nodes (mLNs), where they present

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antigen to naive T cells and, in the presence of IL-4 or IL-6, promote the differentiation of T_H2 or T_H17 cells, respectively.^{20,21} Among the different DC subsets found in the lungs, myeloid CD103[−] signal regulatory protein α (SIRP α)-positive DCs have been identified as the primary immunogenic DC that drives pathogenic T_H2 airway inflammation.²² In the present study we found that repeated local or systemic administration of DCs in the absence of ovalbumin (OVA) aerosol challenge elicits severe airway inflammation and promotes the development of IL-17[−], IL-4[−], and IL-13[−]-producing T_H cells that coexpress GATA-3 and retinoic acid receptor-related orphan receptor (ROR) γ t (T_H17/T_H2) in lungs and mLN. Our *in vitro* studies further suggest that T_H17 cells and not T_H2 cells are reprogrammed into functional T_H17/T_H2 cells by lung inflammatory DCs.

METHODS

Mice

Female BALB/c mice were purchased from Charles River (Wilmington, Mass), and BALB/c DO11.10 T-cell receptor and CD45.1 transgenic mice were purchased from Jackson Laboratory (Bar Harbor, Me). Six- to 8-week-old mice were maintained under specific pathogen-free conditions. All the experimental protocols were approved by the Ethics Committee of Centre Hospitalier de l'Université de Montréal, Research Center and the Canadian Council on Animal Care.

Murine model of airway inflammation

BALB/c mice were sensitized by means of the intratracheal or intravenous injection of 1×10^6 PBS- or OVA-pulsed (10 μ g/mL; Grade V; Sigma, St Louis, Mo) bone marrow-derived dendritic cells (BMDCs). BMDCs (CD11c⁺, CD11b⁺, MHC class II⁺, and SIRP α ⁺) were generated as previously described.^{22,23} Mice were challenged at days 12 and 15 with the respective DCs used for immunization and killed 1 or 3 days later.

Airway histology

Lungs were frozen in OCT compound, and 5- μ m sections were prepared for hematoxylin and eosin and periodic acid-Schiff staining and examined by means of light microscopy.

Cytokine and immunoglobulin measurements

For lung explants, 1 lung lobe was cut into small pieces and cultured for 24 hours in culture medium supplemented with 10% FBS, 500 U/mL penicillin, 500 μ g/mL streptomycin, 10 mmol/L HEPES buffer, and 1 mmol/L 2-mercaptoethanol. mLN cells (4×10^6 cells/mL) were cultured with OVA (100 μ g/mL) for 72 hours in culture medium. IL-4, IL-5 (BD Biosciences, Mississauga, Ontario, Canada), IL-6, IL-13, IL-17, IL-25, and CCL11/eotaxin (R&D Systems, Minneapolis, Minn) levels were measured in mLN and lung culture supernatants by means of ELISA. Total IgE production was quantified in serum by means of ELISA.

Flow cytometric analysis

Total bronchoalveolar lavage fluid cells were stained with anti-CCR3 (R&D Systems), anti-CD3 (clone 145-2C11), and anti-B220 (R&D Systems), as described by van Rijt et al.²⁴ Cytokine expression in CD4⁺ T cells was measured by means of intracytoplasmic staining after restimulation with phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (500 ng/mL) in the presence of brefeldin (10 μ g/mL) for 5 hours. Cells were stained for extracellular markers (anti-CD4, BD Biosciences), fixed, permeabilized, and stained with the following intracellular markers: IL-13, IL-17, IL-4, IFN- γ (BioLegend, San Diego, Calif), and/or GATA-3 (eBioscience, San Diego, Calif). All data were acquired on a FACSaria (BD Biosciences).

DC Sorting

Lungs were treated with Liberase (0.4 mg/mL; Roche, Mannheim, Germany), and lung cell suspensions were stained with the following antibodies: anti-CD4 (BD), anti-CD45.1, anti-CD45.2 (BioLegend), anti-CD11c (BD Biosciences), anti-CD11b (Caltag, Burlingame, Calif), anti-SIRP α , and anti-I-Ad/I-Ed (BD Biosciences). Small nongranular CD4⁺ cells and CD11c^{high}CD11b^{high}SIRP α ^{high} were sorted as T cells and DCs, respectively.

Cocultures

For cocultures, OVA-loaded BMDCs (OVA-DCs) or T cell-depleted splenocytes (OVA-antigen-presenting cells [APCs]) were cultured with purified, naive CD4⁺ transgenic T cells (EasySep; StemCell Technologies, Vancouver, British Columbia, Canada) under T_H2-polarizing (20 ng/mL IL-4, 10 μ g/mL anti-IFN- γ and anti-IL-12, and 1 μ g/mL LPS) or T_H17-polarizing (20 ng/mL IL-6 and IL-23, 1 ng/mL TGF- β , and 10 μ g/mL anti-IFN- γ) conditions. After 5 to 7 days, effector T cells were restimulated with OVA-DCs or OVA-APCs for another 3 to 4 days. For some experiments, sorted CD45.1 (donor) and CD45.2 (host) DCs were cocultured with either T_H2- or T_H17-polarized cells or FACS-sorted lung CD4⁺ T cells in the presence or absence of OVA peptide (2 μ g/mL). All cocultures were performed at a 1:25 DC/T-cell ratio.

Statistical analysis

Statistical analyses were performed with the unpaired Student *t* test and the nonparametric Mann-Whitney *U* test.

RESULTS

Local or systemic repetitive immunization with DCs induces IgE-independent antigen-specific T_H17 responses

Lung myeloid DCs are sufficient to drive T_H2 and T_H17 responses, which are both implicated in the pathogenesis of allergic disorders.^{12,20} We investigated the effect of repetitive administration of pathogenic SIRP α ⁺ DCs through the intratracheal route on the development of T_H17 and T_H2 responses and airway inflammation. Lung sections of mice immunized with OVA-loaded BMDCs (OVA-DCs) revealed a significant peribronchial accumulation of inflammatory cells, goblet cell hyperplasia, and abundant mucus production (Fig 1, A). The cellular infiltrate was characterized by eosinophils, neutrophils, and lymphocytes (Fig 1, B). In several models of experimental asthma, steroid-resistant airway inflammation has been associated with IgE-independent T_H17-mediated airway responses.¹⁸ We found that serum IgE levels were similar in naive and DC-immunized mice (Fig 1, C) and that the quantity or composition of recruited inflammatory cells in bronchoalveolar lavage fluid was not modified by local steroid administration (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org). OVA-DCs significantly augmented IL-17 production in mLN cells (Fig 1, D), as well as in lung tissues, when compared with unpulsed DCs (PBS-DCs; Fig 1, E), and T_H17 responses were not decreased in steroid-treated immunized mice (see Fig E1, C). However, mice that were sensitized and challenged with PBS-DCs or OVA-DCs expressed similar quantities of IL-4 and IL-13 in mLN and lungs, suggesting that repetitive injection of DCs promoted OVA-independent T_H2 responses (Fig 1, D and E). Lung eotaxin and IL-6 release also occurred in the absence of antigen (Fig 1, E), which might reflect cellular damage provoked by the passage of DCs through the airway epithelial barrier. Epithelial cells represent an important source of IL-25 and thymic stromal

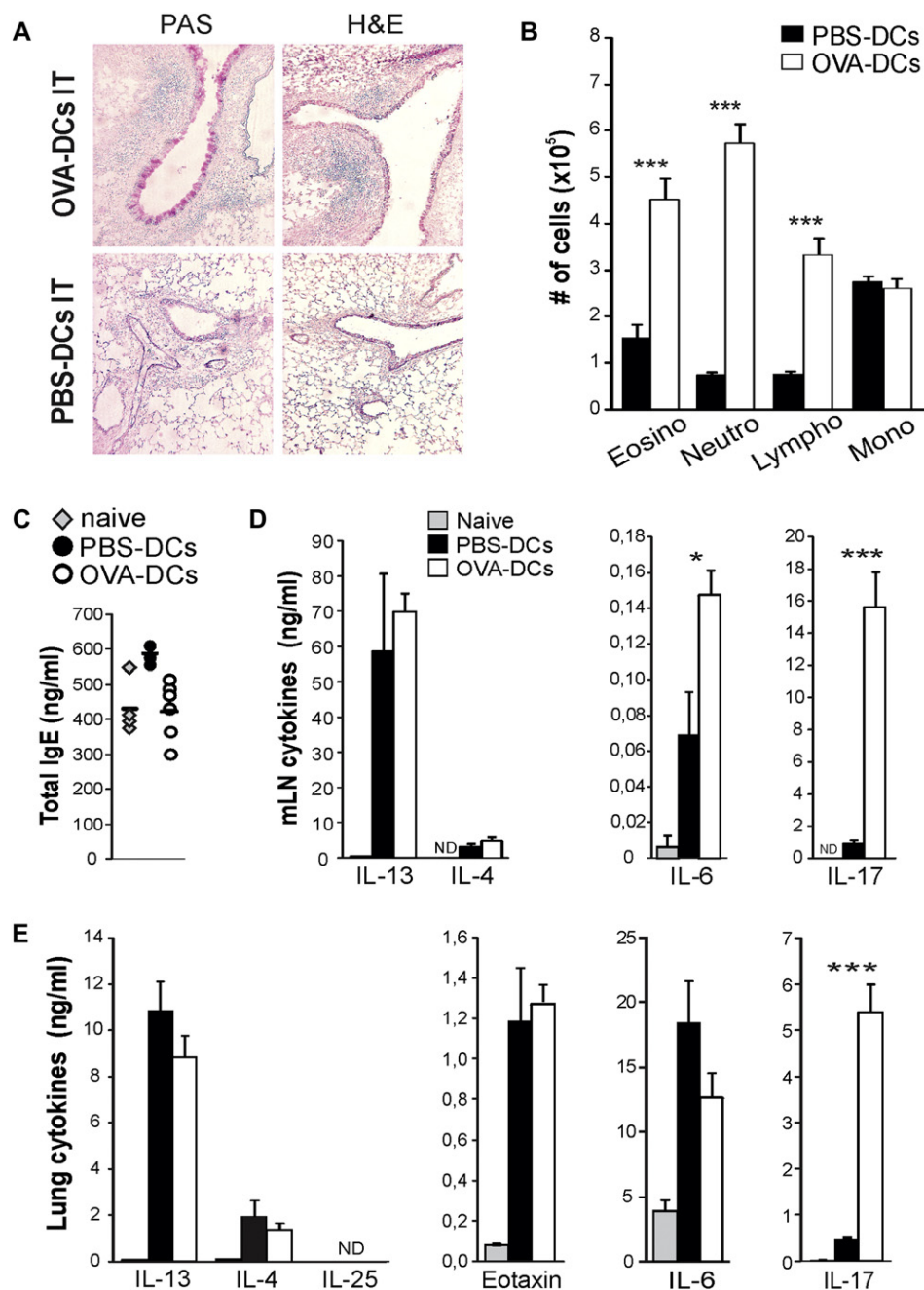


FIG 1. Repetitive local administration of OVA-DCs induces an IgE-independent antigen-specific T_H17 response. Mice were injected with PBS-DCs or OVA-DCs intratracheally (IT) at days 0, 12, and 15. **A**, Lung sections stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). **B**, Differential cell count in bronchoalveolar lavage fluid. **C**, Total IgE level in serum. **D** and **E**, Cytokine release by mLN cells after OVA restimulation *in vitro* (Fig 1, **D**) and lung explants (Fig 1, **E**). Values are presented as means $\pm$ SEMs ($n = 4$ –8 mice per group from at least 2 independent experiments). *** $P < .001$, * $P < .05$.

lymphopoietin.^{25,26} However, neither IL-25 nor thymic stromal lymphopoietin expression was detected in lung tissues (Fig 1, **E**), which might be taken as one indication of the absence of epithelial cell activation in response to repetitive DC administration.

We therefore examined the profile of the antigen-specific response after repetitive systemic injections of DCs (Fig 2). Because the lungs can act as a blood filter, the intravenous route allowed DCs to be trapped in the lungs and to be recruited as

early as day 2 to the mLNs (see Fig E2 in this article's Online Repository at www.jacionline.org). At the same time, the systemic route prevents epithelial damage, a primary event that drives pulmonary T_H2 or T_H17 responses.²⁷ Repetitive intravenous immunization with OVA-DCs induced the recruitment of neutrophils and lymphocytes in the bronchoalveolar lavage fluid, with no significant increase in eosinophil counts, monocyte counts, or serum IgE levels when compared with those

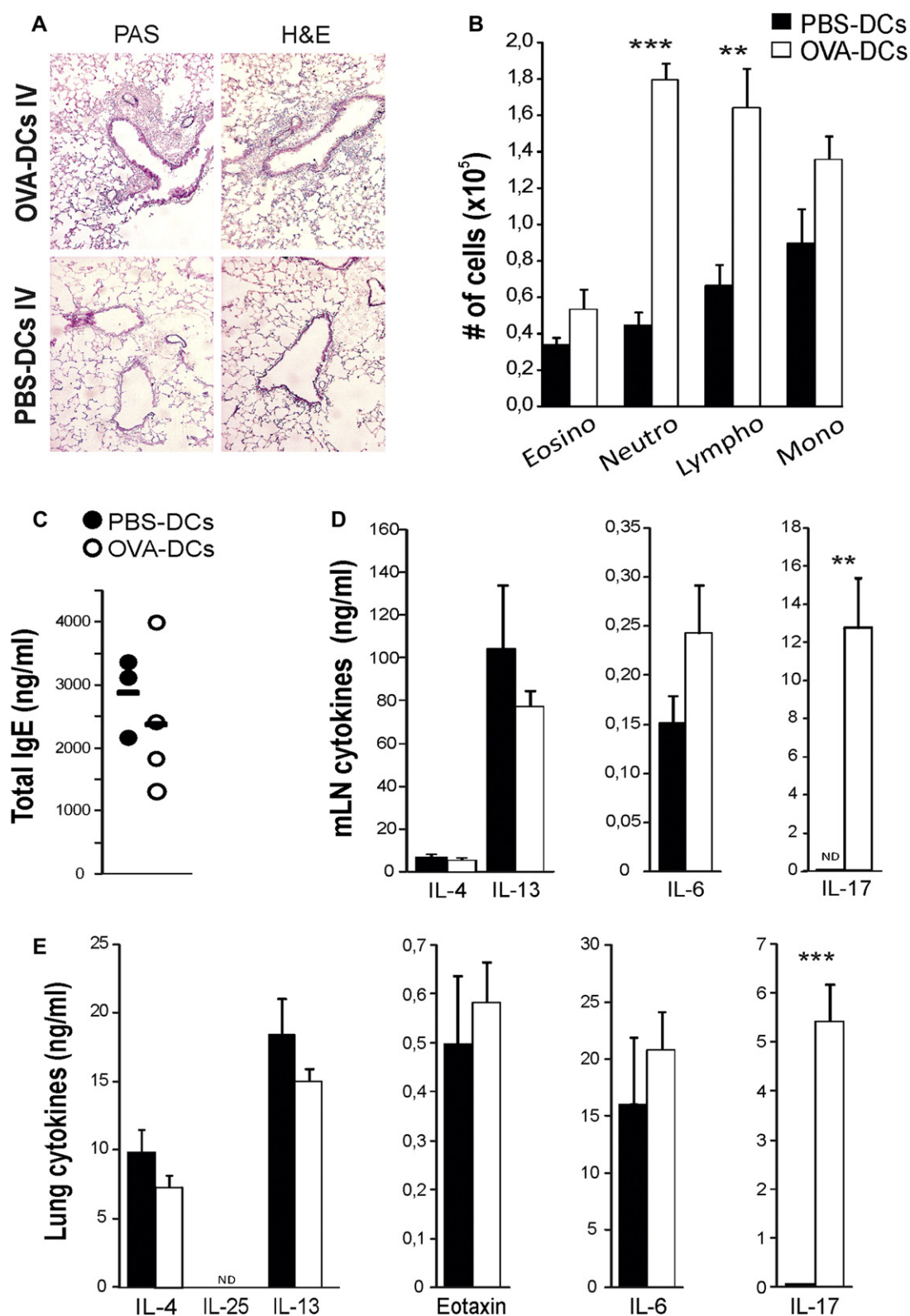


FIG 2. Systemic injection of OVA-DCs induces antigen-specific T_H17 -associated airway inflammation. Mice were injected with PBS-DCs or OVA-DCs intravenously (IV) at days 0, 12, and 15. **A**, Lung sections stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). **B**, Differential cell count in bronchoalveolar lavage fluid. **C**, Total IgE level in serum. **D** and **E**, Cytokine release by mLN cells after OVA restimulation *in vitro* (Fig 2, D) and lung explants (Fig 2, E). Values are presented as means $\pm$ SEMs ($n = 4$ -7 mice per group from at least 2 independent experiments). *** $P < .001$, ** $P < .01$.

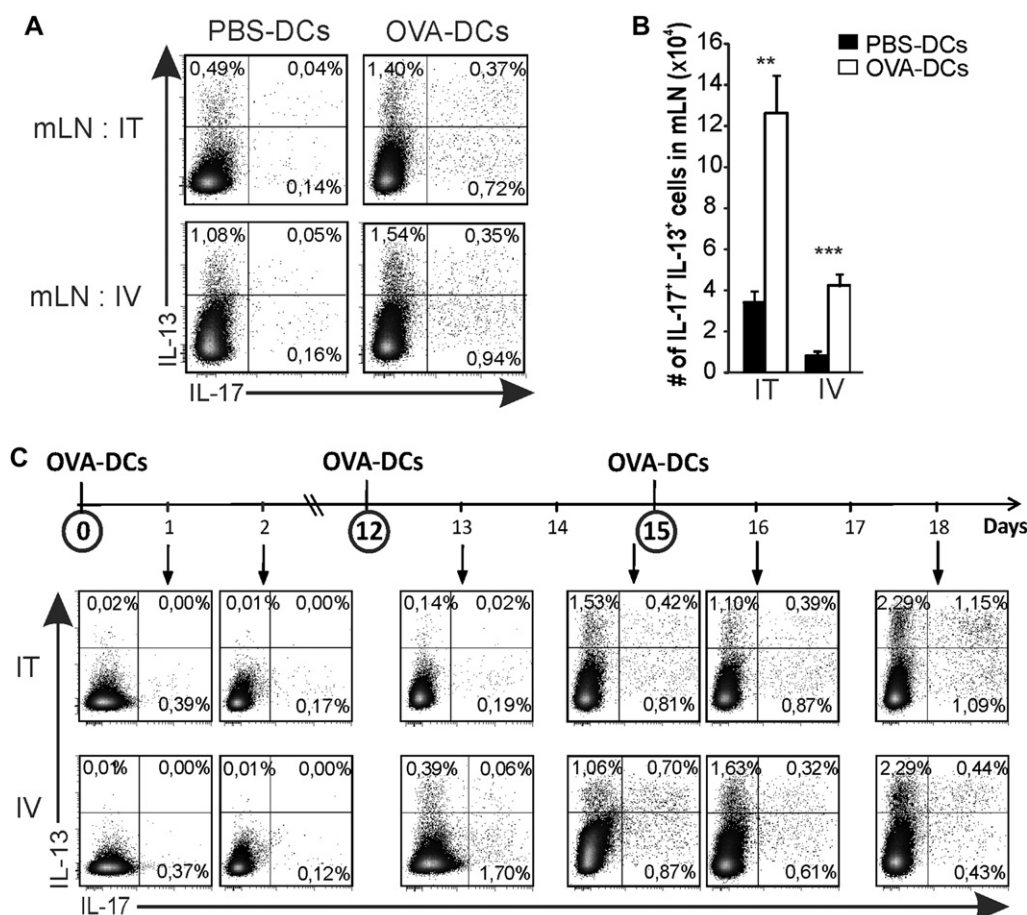


FIG 3. Local and systemic administration of inflammatory OVA-DCs induces double IL-17⁺IL-13⁺-producing T cells in mLN. Mice were injected with PBS-DCs or OVA-DCs intratracheally (IT) or intravenously (IV) at days 0, 12, and 15. **A**, IL-17 and IL-13 expression of CD4⁺ T cells in mLN. **B**, IL-17⁺IL-13⁺-producing CD4⁺ T cells in mLN. Values are presented as means ± SEMs (n = 4–7 mice per group from at least 2 independent experiments). **C**, IL-17/IL-13 expression in mLN (gated on CD4⁺ T cells) at different time points. Dot plots (Fig 3, A and C) show 1 representative experiment of 3 independent experiments (at least 3 mice per group). ***P < .001, **P < .01.

seen in PBS-DC-immunized and challenged mice (Fig 2, B and C). The peribronchial and perivascular lung cellular infiltration was observed at a lower intensity than in mice immunized with DCs through the intratracheal route (Fig 2, A). However, mice sensitized and challenged with OVA-DCs through the intravenous and intratracheal route displayed similar quantities of antigen-specific IL-17 production by mLN cells and lung cells (approximately 5 to 10 ng/mL). As for intratracheal immunization, the T_H2 response appeared to be antigen independent because intravenous repetitive administration of PBS-DCs was sufficient to trigger IL-4, IL-13, and eotaxin release (Fig 2, D and E).

Therefore, OVA-DCs drive an IgE-independent antigen-specific T_H17 response associated with mixed neutrophilic/eosinophilic airway infiltration, although dominated by neutrophilic inflammation, independently of the route of immunization (see Fig E3 in this article's Online Repository at www.jacionline.org). These data provide evidence that epithelial cell damage after exposure to antigen is not necessarily the obligatory primary event to initiate a local antigen-specific T_H17 response and airway inflammation.

Local or systemic DC administration induces the differentiation of CD4⁺ T cells that produce IL-17 and IL-13 *in vivo*

We next showed that immunization (intratracheal or intravenous) with OVA-DCs increased the frequency of CD4⁺ T cells that produced IL-17 and induced a substantial proportion of IL-17⁺IL-13⁺CD4⁺ T cells in mLN (Fig 3, A). We observed a 4-fold (intratracheal) to 5-fold (intravenous) increase in the numbers of double-positive cells in the OVA-restimulated mLN cells in response to immunization with OVA-DCs when compared with PBS-DCs (Fig 3, B). Repeated immunization with OVA-DCs, independent of the immunization route, promoted a temporal change in the quality of the T_H responses (from single to double IL-17⁺IL-13⁺ CD4⁺ T cells), which correlated with airway inflammation. In fact, a resting period of 3 days after the second immunization was necessary and sufficient to drive airway inflammation and a mixed T_H17 and T_H2 response because double-positive IL-17/IL-13-secreting CD4⁺ T cells were not detected before day 15 (Fig 3, C). In agreement with a recent report,²⁸ lung inflammation and, as depicted in Fig E4 in this article's Online Repository at www.jacionline.org.

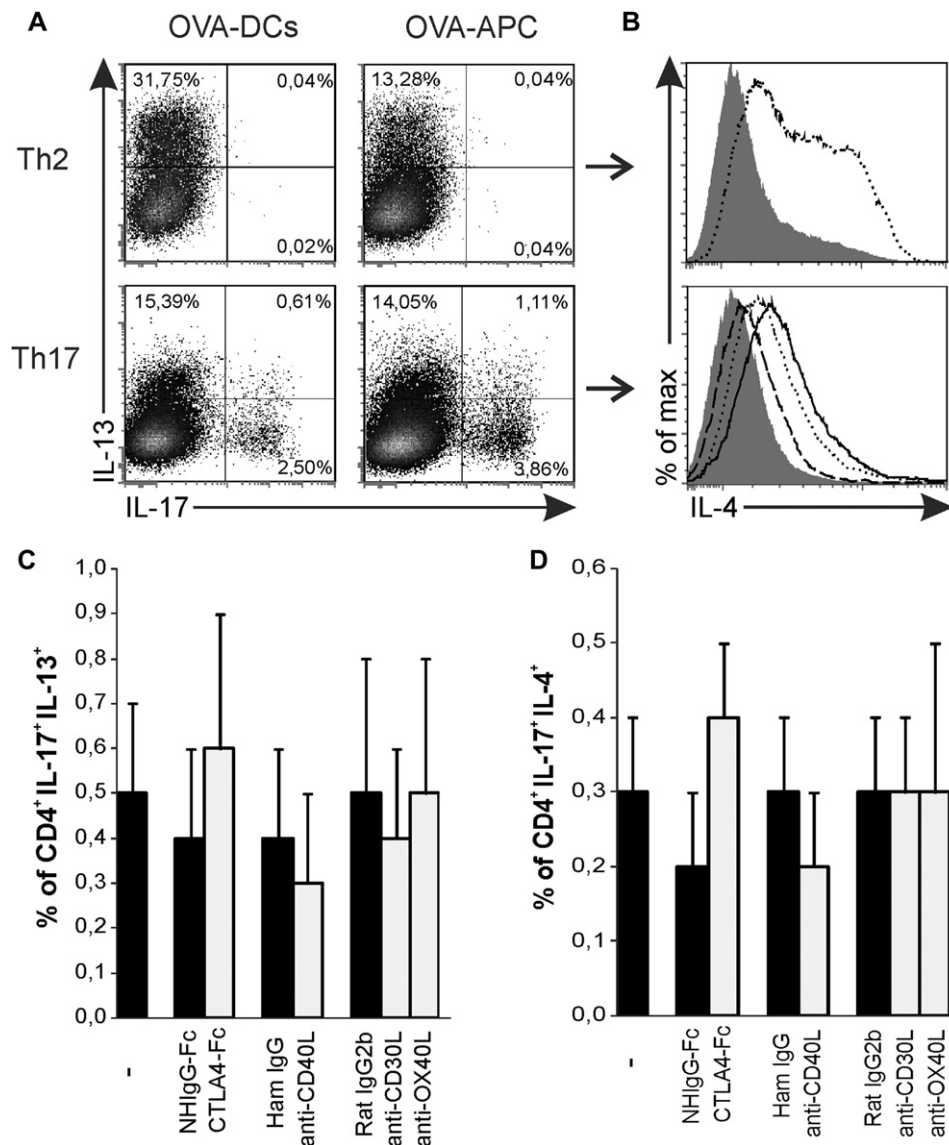


FIG 4. *In vitro* conversion of T_H17 - but not T_H2 -polarized T cells into T_H17/T_H2 cells. **A**, Transgenic $CD4^+$ T cells were polarized *in vitro* into T_H2 (upper panel) or T_H17 (lower panel) cells. T_H cells were restimulated with OVA-DCs (left panel) or OVA-APCs (right panel) and examined for IL-13 and IL-17 expression. **B**, IL-4 expression in IL-17⁻IL-13⁻ (filled histogram), IL-17⁺IL-13⁻ (dashed line), IL-17⁻IL-13⁺ (dotted line), and IL-17⁺IL-13⁺ (full line) T_H cells. Data are from 1 representative experiment of 2 independent experiments. **C** and **D**, OVA-DCs were cocultured with T_H17 cells in different conditions. Values are presented as means $\pm$ SEMs of 4 independent experiments. *CD30L*, CD30 ligand; *CD40L*, CD40 ligand; *CTLA4*, cytotoxic T lymphocyte-associated antigen 4; *Ham*, hamster; *NHlgG*, normal human IgG; *OX40L*, OX40 ligand.

org, IL-17⁺IL-13⁺CD4⁺ T cells were not initiated by antigen protein in the absence of infection (ie, DCs loaded with endotoxin-free OVA protein).

IL-17⁺CD4⁺ T cells producing IL-13 and IL-4 are generated *in vitro* from established T_H17 but not T_H2 cells

We next determined whether priming and restimulation with OVA-loaded APCs would reprogram established T_H17 or T_H2 cell lines into T_H17/T_H2 *in vitro*. Double IL-17⁺IL-13⁺CD4⁺ T cells were exclusively detected after restimulation of

polarized T_H17 cells independently of the quality of the APCs used for primary and secondary stimulation (Fig 4, A). Additionally, IL-17⁺IL-13⁺ T cells expressed IL-4, pointing to the plasticity of *in vitro*-generated T_H17 cells and their ability to acquire a T_H2 phenotype (Fig 4, B). The costimulatory molecules that are critical for T_H2 differentiation, activation, or both were not implicated in the generation of IL-17⁺IL-13⁺IL-4⁺CD4⁺ T cells (T_H17/T_H2) *in vitro* because cytotoxic T lymphocyte-associated antigen 4-Ig fusion protein, neutralizing anti-CD40 ligand, anti-CD30 ligand, and anti-OX40 ligand mAbs had no effect on the proportion of T_H17/T_H2 cells (Fig 4, C and D).

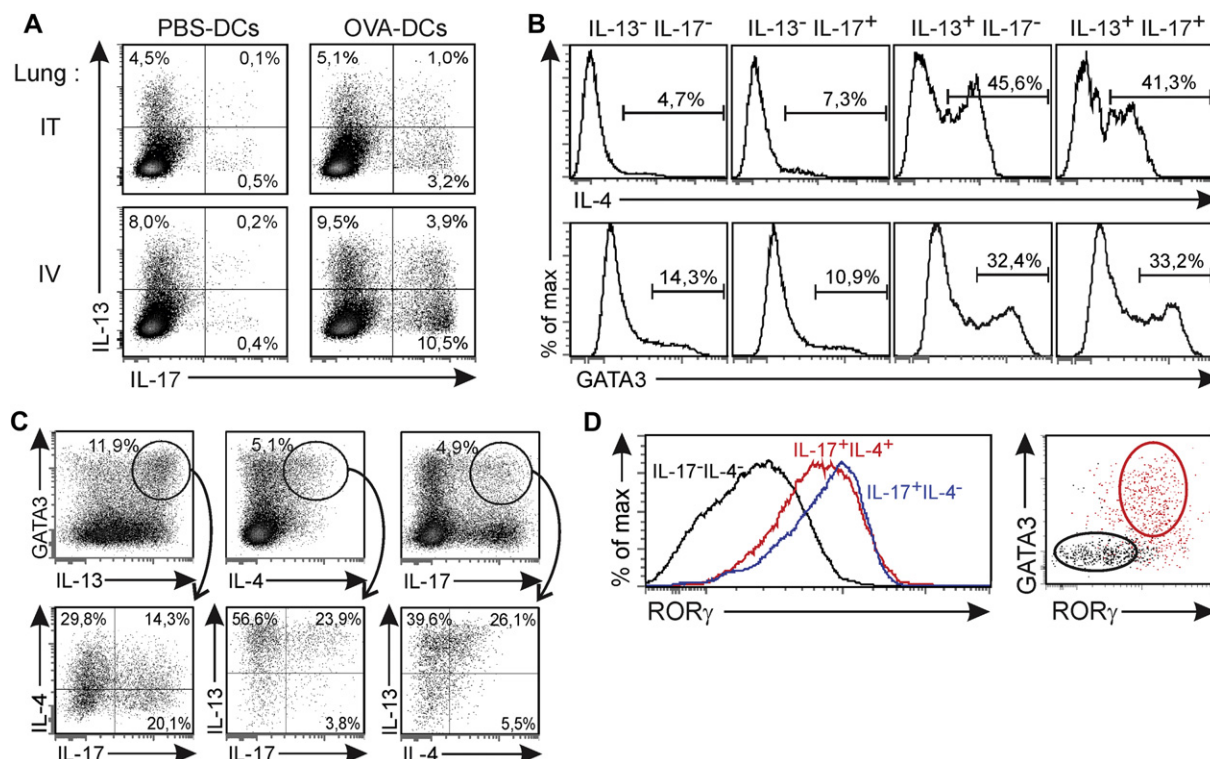


FIG 5. Identification of IL-17⁺IL-13⁺IL-4⁺CD4⁺ T cells (T_H17/T_H2) that coexpress GATA-3 and RORγt in inflamed lung tissues. Mice were injected with PBS-DCs or OVA-DCs intratracheally (IT; Fig 5, A) or intravenously (IV; Fig 5, A-C) at days 0 and 12 and killed on day 15. **A**, IL-13 and IL-17 expression among CD4⁺ T cells in lung tissues. **B**, IL-4 or GATA-3 expression according to IL-13/IL-17 profile. **C**, IL-4, IL-13, and IL-17 coexpression in CD4⁺GATA-3⁺ T cells. **D**, RORγt and GATA-3 coexpression in IL-17⁺IL-4⁺CD4⁺ T cells. Data are from 1 representative experiment of 2 independent experiments (3 mice per group).

Triple IL-17⁺IL-13⁺IL-4⁺CD4⁺ T cells that coexpress RORγt and GATA-3 are found in the lung tissues of mice locally or systemically immunized with inflammatory DCs

We next demonstrated the presence of IL-17⁺IL-13⁺CD4⁺ T cells in the lungs of mice that were immunized/challenged with OVA-DCs (Fig 5, A). After 3 days of rest, the proportion of double-positive cells was increased in the airways when compared with the frequency seen in mLNs (Fig 3, C). The expression pattern of the T_H2 transcription factor GATA-3 correlated with that of IL-4 because more than 30% of single IL-13⁺ or double IL-17⁺IL-13⁺ pulmonary effector CD4⁺ T cells expressed GATA-3 or IL-4 (Fig 5, B). In fact, the great majority of IL-17⁺ T cells coexpressed IL-13 in the IL-4⁺GATA-3⁺ T-cell subpopulation, and more than 90% of IL-4⁺ T cells coexpressed IL-13 in IL-17⁺GATA-3⁺ T cells (Fig 5, C). IL-17⁺IL-4⁺ T cells coexpressed GATA-3 and RORγt, although at a lower intensity than committed T_H17 cells (Fig 5, D). IFN-γ expression was not detected in IL-17⁺IL-13⁺GATA-3⁺ T cells (see Fig E5 in this article's Online Repository at www.jacionline.org). Together, these data demonstrate the existence of T_H17/T_H2 cells that coexpress GATA-3 and RORγt in lung tissues of repeatedly immunized mice with chronic airway inflammation.

Airway inflammatory DCs convert lung effector T cells into T_H17/T_H2 cells that express GATA-3

We finally investigated the cellular mechanisms that govern the induction of triple IL-17⁺IL-13⁺IL-4⁺ T cells in lung

tissues. We asked whether pulmonary DCs isolated from repetitively immunized mice could provide help *in vitro* to induce IL-13 expression in established T_H17 cells. To this end, OVA-DCs were generated from congenic CD45.1 mice and used to sensitize and challenge CD45.2 hosts. Donor (CD45.1) and host (CD45.2) CD11c^{high}CD11b^{high}SIRPα^{high} airway DCs were purified 1 day after OVA-DC challenge and cocultured with established T_H17 or T_H2 cell lines (Fig 6, A). Donor and recipient airway DCs, as well as *in vitro*-generated donor OVA-DCs, reprogrammed T_H17 cells but not T_H2 cells into T_H17/T_H2 cells. We next examined whether host airway DCs were sufficient or required to convert effector CD4⁺ T cells isolated from lungs of CD45.2 hosts into T_H17/T_H2 cells. As observed in mLNs on day 13 (Fig 3, C), almost no double IL-17⁺IL-13⁺ cells were detected in *ex vivo*-isolated lung effector CD4⁺ T cells 1 day after the OVA-DC challenge, and those CD4⁺ T cells maintained their phenotype when cocultured in the absence of DCs (Fig 6, B, left panel). Cocultures of lung effector T cells with *in vitro*-generated OVA-DCs or purified host airway DCs promoted the generation of double IL-17⁺IL-13⁺CD4⁺ T cells that expressed IL-4 and GATA-3 (Fig 6, B, right panel, and see Fig E6 in this article's Online Repository at www.jacionline.org). We noticed that the addition of OVA was not required for inflammatory host DCs to drive T_H17/T_H2 responses. Our findings thus indicate that host-derived inflammatory airway DCs are sufficient to convert lung effector T_H cells into T_H17/T_H2 cells.

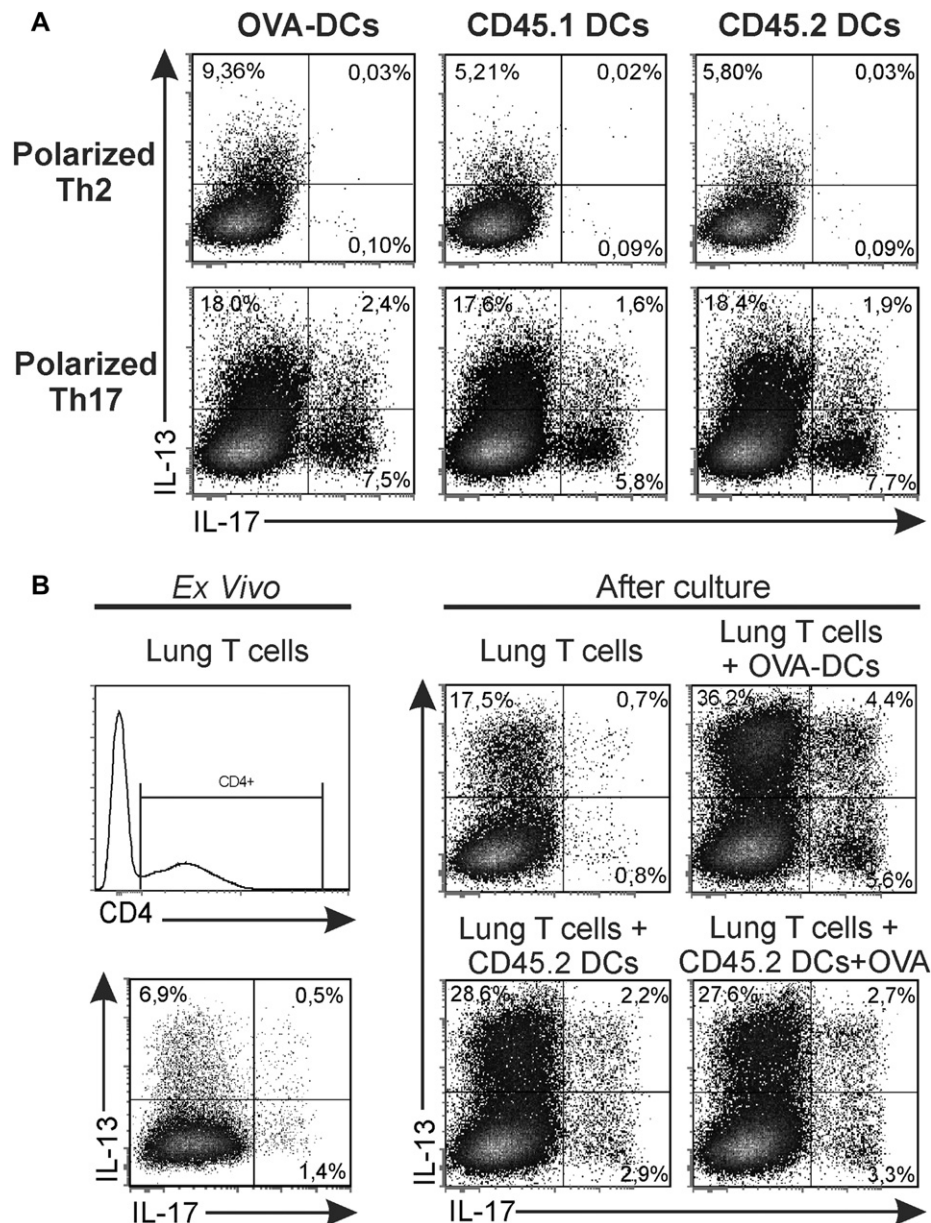


FIG 6. Airway host DCs convert effector CD4⁺ T cells into TH17/TH2 cells *in vitro*. CD45.2 mice were injected with OVA-CD45.1 DCs intratracheally or intravenously at days 0 and 12 and killed on day 15. OVA-DCs (CD45.1 DCs before injection) or FACS-sorted donor (CD45.1) and host (CD45.2) airway DCs were cocultured with TH2- or TH17-polarized cells (**A**) or lung effector CD4⁺ T cells (**B**). IL-13 and IL-17 expression in CD4⁺ T cells before (*ex vivo*) or after culture. Data are from 1 representative experiment of 4 or 3 independent experiments.

DISCUSSION

The conversion of CD4⁺ TH cell subsets has relied mostly on extensive *in vitro* manipulation.⁶ It is thought that murine TH17 memory cells generated *in vivo* maintain a stable phenotype and cannot be manipulated *in vitro* to express a TH2 cytokine profile.²⁹ However, evidence of the plasticity of TH17 cells toward TH2 cells is provided by the presence of IL-17⁺IL-4⁺ TH cells in *ex vivo*-isolated peripheral blood of healthy donors.³⁰ In the present study we demonstrated that inflammatory airway DCs drive the generation of TH17 cells that produce IL-4 and IL-13 in the mLN and lung tissues. Repeated immunization of mice with inflammatory OVA-DCs elicited antigen-specific TH17/TH2 lung T

cells that coexpress GATA-3 and RORγt. We further showed that established TH17 cells were converted *in vitro* by inflammatory DCs into IL-13⁺ and IL-4⁺-expressing cells, whereas TH2 cell lines did not express IL-17 under similar culture conditions, suggesting that TH17 cells were reprogrammed to produce TH2 cytokines and not vice versa. Finally, we provided evidence that repetitive injection of inflammatory OVA-DCs prevails on the route of immunization (ie, systemic or local) to drive TH17/TH2 responses and chronic lung inflammation.

An increasing body of evidence has revealed that the route of immunization affects the quality and cytokine profile of CD4⁺ TH cell responses. Sensitization through the peritoneum with

adjuvant followed by repeated airway challenge induces pulmonary IgE-dependent T_H2 responses and no T_H17 responses, whereas airway allergic sensitization promotes neutrophil-dependent T_H17 responses.³¹ In another study systemic immunization elicits a T_H2 or T_H1 bias in response to infectious agents, whereas the short-term T_H17 memory response is driven by intranasal immunization.³² The environment in mucosal tissues, an enriched TGF- β milieu, is propitious for the differentiation of IL-17-producing cells, which require both IL-6 and TGF- β to develop.³³ We showed that the repetitive intravenous injection of OVA-DCs induced antigen-specific T_H17 responses in the mLN and lung tissue in the absence of an airway challenge, indicating that the passage through the epithelial barrier is not an obligatory route to initiate a pulmonary T_H17 response. Indeed, OVA-DCs injected intravenously rapidly migrated to the lungs and mLN. The notion that tissue-resident DCs could reach the blood and recirculate has surfaced repeatedly.³⁴ Circulating DCs continuously access bone marrow, where they can activate resident memory T cells.³⁵ Transcutaneous sensitization elicits T-cell responses at distal mucosal sites, suggesting that skin DCs collect antigen in the periphery and traffic to the inductive site to activate T cells. This does not exclude the possibility that DCs migrate to LN to activate T cells, which are known to return to the effector sites.³⁶ Epicutaneous antigen delivery drives T_H17 airway responses on OVA inhalation, even in the absence of IL-4 and IL-13.³⁷ Finally, a recent concept has emerged that the mucosal immune system can be viewed as a global organ characterized by diverse relationships between mucosal sites with a potential key role for DCs in connecting the different sites.³⁸

T_H1 , T_H2 , and T_H17 cells were first identified as 3 distinct lineages that rely on distinct signals and transcription factors. *Gata3* is selectively induced in developing T_H2 cells after T-cell receptor stimulation in the presence or absence of IL-4, the source of which is still debated.¹¹ Ectopic expression of *Gata3* in developing T_H1 cells upregulates IL-4 and downregulates IFN- γ .³⁹ We found that T_H17/T_H2 cells expressed GATA-3, as well as ROR γ t, the hallmark of committed T_H2 and T_H17 cells, respectively. We further showed that the repetitive administration of OVA-DCs intratracheally or intravenously was required to amplify the T_H17 response, transform it into a T_H17/T_H2 response, and promote chronic airway inflammation. However, unpulsed DCs triggered significant production of IL-6 and IL-13, which was comparable with that induced by OVA-DCs, indicating their ability to induce a local inflammatory response.

The following studies corroborate our findings. First, intratracheal immunization with unpulsed DCs 3 times over a 2-week interval is sufficient to drive the induction and maintenance of bronchial-associated lymphoid tissue⁴⁰ and lung inflammation. Second, consecutive subcutaneous injections of inflammatory DCs 2 days apart induces reactive lymph nodes that become permissive for the development of experimental autoimmune encephalomyelitis after intravenous immunization with MOG peptide 30 days later in the absence of adjuvant.⁴¹

Mature nonplasmacytoid DCs are important to the development of T_H17 -mediated airway disease. Lung mature nonplasmacytoid DCs promote the generation of pathogenic T_H1 and T_H17 responses and favor the maintenance of a destructive inflammatory state in T_H17 -associated lung emphysema.⁴² Also, lung DCs exposed to house dust mite or BMDCs loaded with cockroach antigen produce IL-6, which drives T_H2 and T_H17 responses, as well as airway inflammation.^{21,43} We showed that

FACS-sorted donor OVA-DCs, as well as inflammatory host airway DCs, were sufficient to promote IL-17, IL-13, and IL-4 coexpression in lung effector T cells. The transfer of antigen between donor and host airway DCs appears to play a minimal role in the induction of robust T_H cell priming,⁴⁴ suggesting that OVA-DCs delivered intratracheally were the primary initiators of naive T_H cell polarization in the mLN.

It was proposed that T_H17 and T_H2 cooperate to elicit lung inflammation and bronchial hyperreactivity. First, administration of IL-17, but not neutrophil-attracting chemokines, is required to induce asthma features in mice that had been immunized systemically (intraperitoneally) and display an ongoing T_H2 response.³¹ Second, adoptive transfer of antigen-specific T_H17 cells alone initiates neutrophil recruitment in the airways, whereas cotransfer of T_H17 and T_H2 cells promotes a mixed neutrophilic and eosinophilic infiltration.^{8,45} Third, strains of mice with severe asthma produce both IL-17 and T_H2 cytokines, whereas resistant mice produce IL-13 and little IL-17.⁴⁶ Fourth, the proportion of IL-17⁺IL-4⁺CD4⁺ T cells seen at a low frequency in the circulation of healthy donors is increased in patients with severe asthma.^{8,30}

Precisely how nonallergic asthma is initiated, why it persists, and how T_H17 , T_H2 , or T_H17/T_H2 effector cells are implicated in disease pathogenesis remain to be determined.⁴⁷ Taken together, we propose that lung DCs that have captured antigen from the external world (airway sensitization) or at a peripheral site (systemic or epicutaneous immunization) initiate a temporal sequence of immune responses in the lung, which are characterized by a mixed neutrophilic/eosinophilic inflammation. Airway inflammatory DCs subsequently migrate to the mLN whereby they prime antigen-specific T_H17 responses that are reprogrammed into T_H17/T_H2 responses in the mLN, on return to the lung tissues, or both. In that context interfering with DC migration/function remains a promising therapeutic option for IgE-independent, steroid-resistant severe asthma.

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Key messages

- Local or systemic DC administration induces T_H17/T_H2 cell-associated, steroid-resistant airway inflammation.
- Lung inflammatory SIRP α^+ DCs can convert lung T_H17 cells into T_H17/T_H2 cells *in vitro* and *in vivo*.
- Interfering with DC migration/function remains a promising therapeutic option for IgE-independent, steroid-resistant severe asthma in human subjects.

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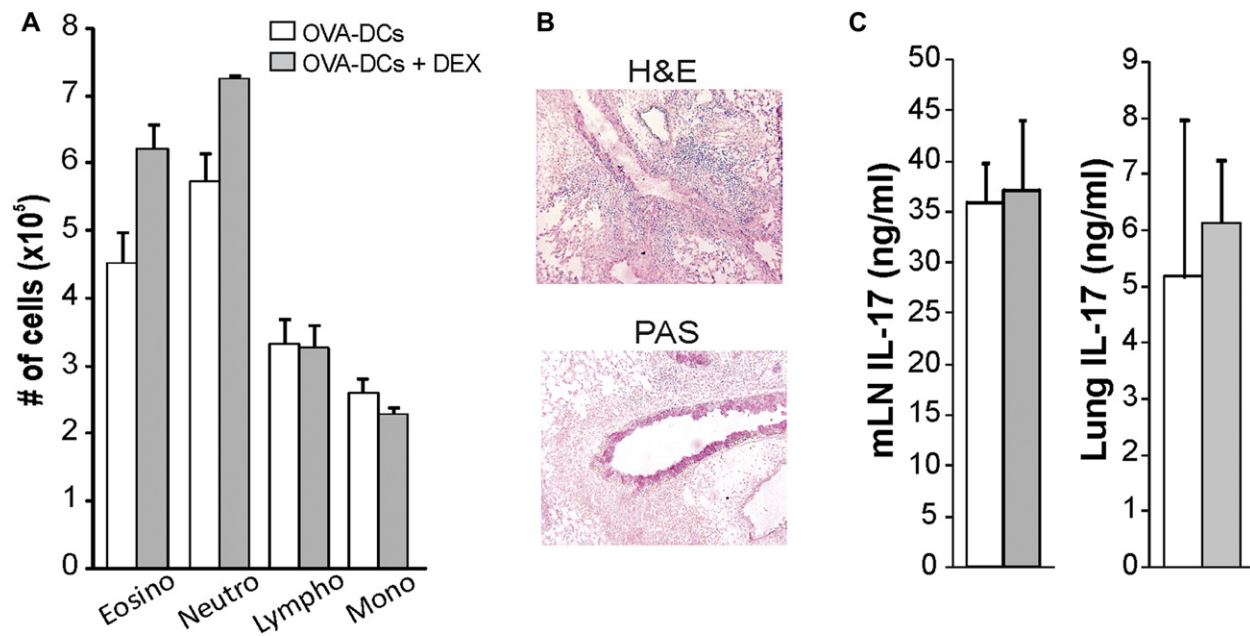


FIG E1. Repetitive local injection of OVA-DCs induces corticosteroid-resistant T_H17 airway inflammation. Mice were injected with PBS-DCs or OVA-DCs intratracheally at days 0, 12, and 15. Some mice were treated daily with dexamethasone (DEX; 2.5 mg/kg administered intraperitoneally) at days 12 to 15. **A**, Differential cell count in bronchoalveolar lavage fluid. **B**, Lung sections stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). **C**, Cytokine release by mLN cells after *in vitro* restimulation and lung explants. Values are presented as means $\pm$ SEMs of 6 mice per group.

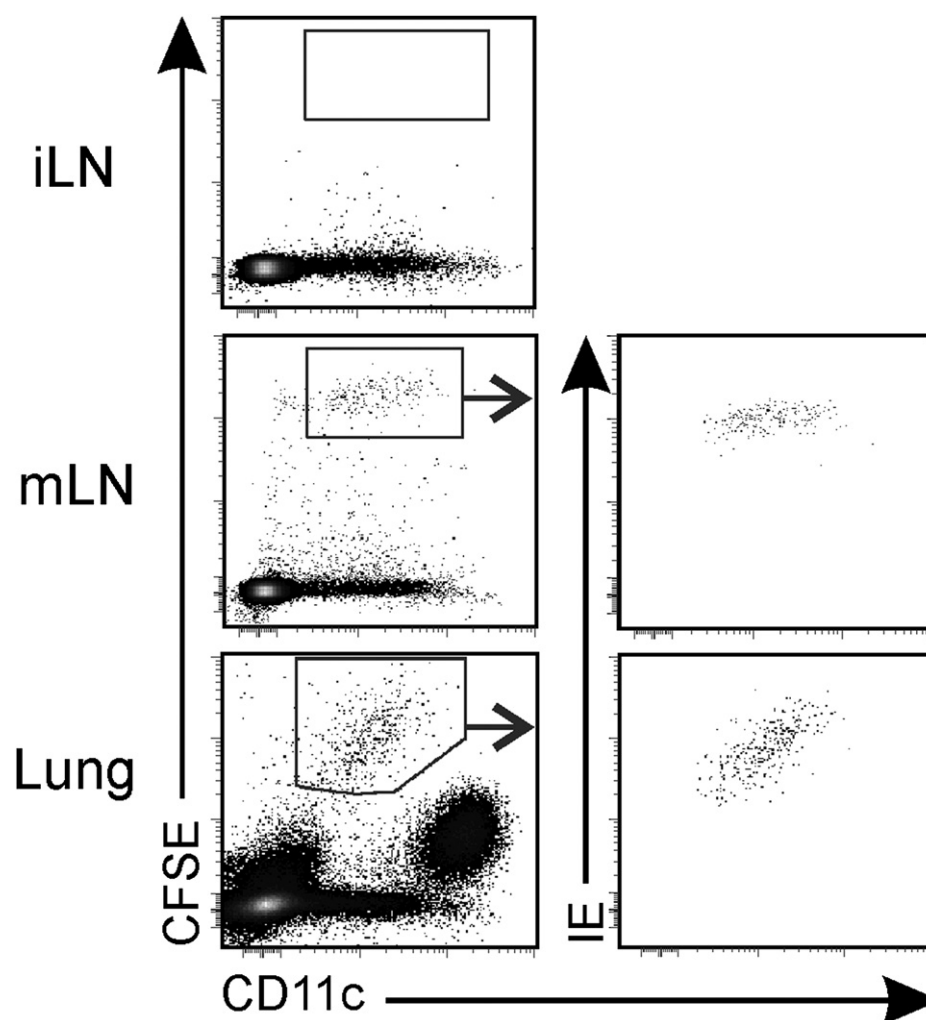


FIG E2. *In vivo* trafficking to lungs and mLNs of DCs injected intravenously. Carboxyfluorescein succinimidyl ester-labeled OVA-DCs were injected intravenously, and mice were killed 48 hours later. Inguinal lymph nodes (*iLN*), mLNs, and lung tissue were examined for the detection of carboxyfluorescein succinimidyl ester-labeled CD11c⁺ MHC class II⁺ DCs. Data represent 1 representative experiment of 2 experiments. *IE*, Anti-I-Ad/I-Ed.

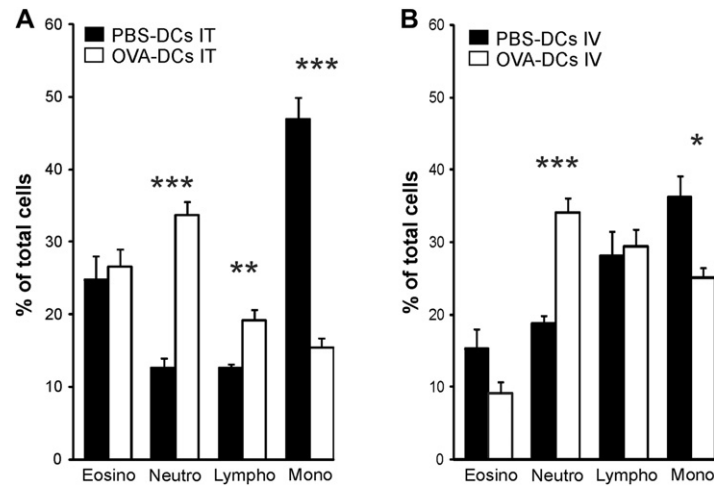


FIG E3. Repetitive local or systemic administration of OVA-DCs induces neutrophil recruitment to airways. Mice were injected with PBS-DCs or OVA-DCs intratracheally (A) or intravenously (B) at days 0, 12, and 15. Differential proportion of infiltrating cells in bronchoalveolar lavage fluid is shown. Values are presented as means $\pm$ SEMs ($n = 4-8$ mice per group from 2 independent experiments). *** $P < .001$, ** $P < .01$, * $P < .05$.

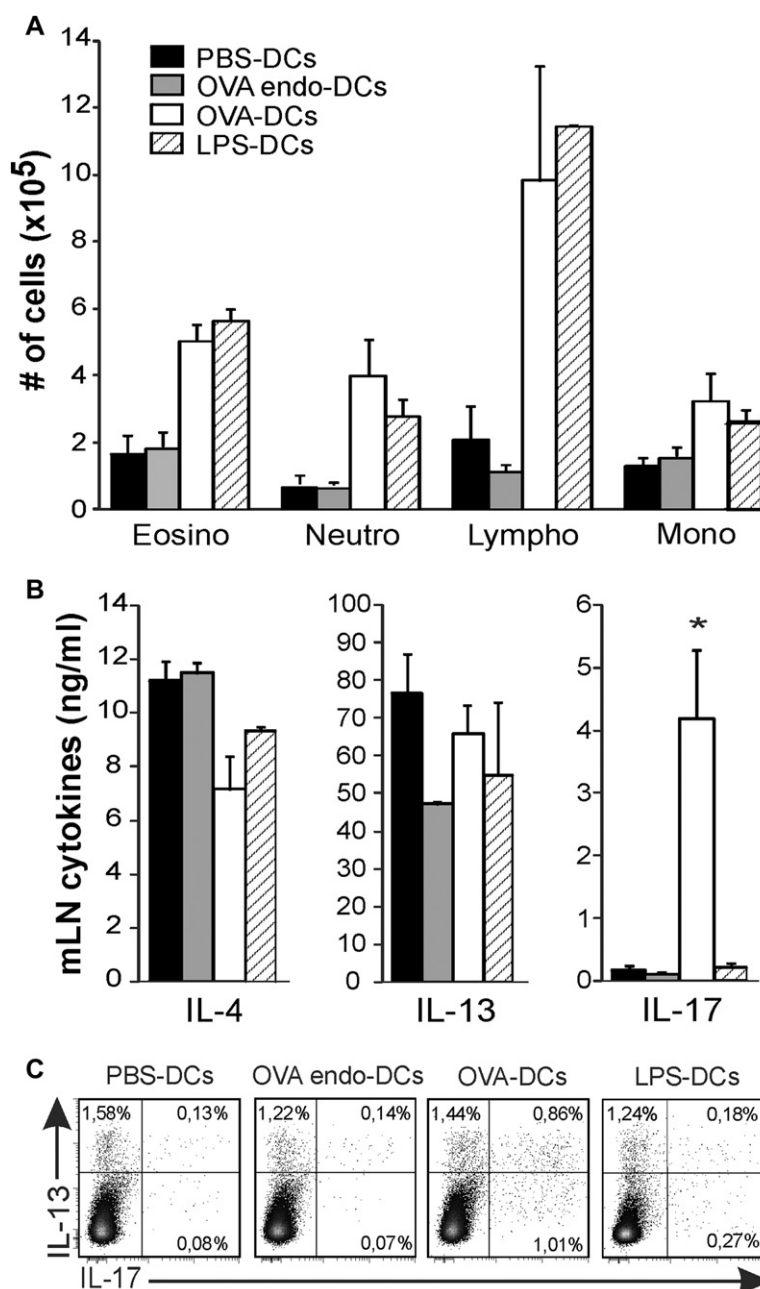


FIG E4. Inflammatory DCs and T-cell receptor stimulation are necessary to initiate T_H17 responses. Mice were sensitized intravenously at days 0 and 12 with PBS-DCs, OVA-DCs, endotoxin-free OVA-DCs (*OVA endo-DCs*), or low-dose LPS-DCs. Mice were killed on day 15. **A**, Differential cell count in bronchoalveolar lavage fluid. **B**, Cytokine release in mLNs after OVA restimulation *in vitro*. Data are presented as means $\pm$ SEMs of 3 to 5 mice per group. **C**, IL-17/IL-13 expression in CD4⁺ T cells. Dot plots represent 1 representative of at least 3 mice per group. * $P < .05$.

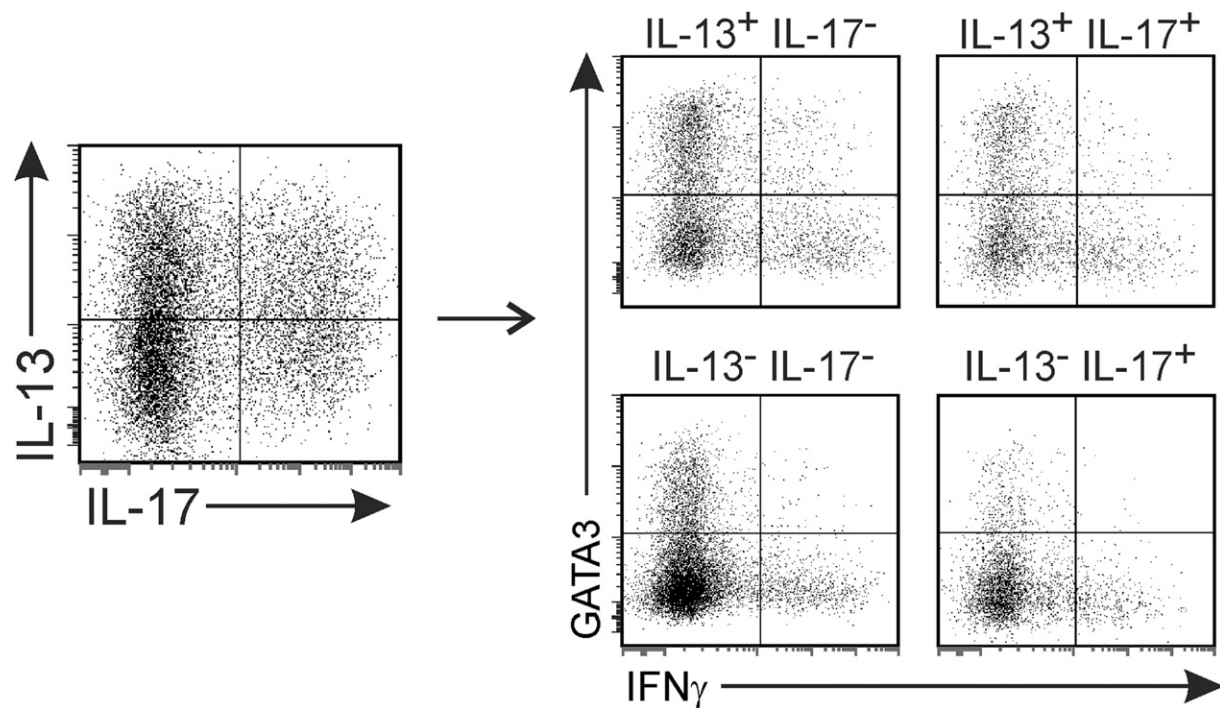


FIG E5. $T_H17^+T_H2^+$ lung $CD4^+$ T cells that express GATA-3 do not produce IFN- γ . Mice were injected with OVA-DCs intravenously at days 0 and 12 and killed on day 15. IL-13 and IL-17 expression among $CD4^+$ T cells in lung tissues (*left panel*) and IFN- γ and GATA3 expression according to IL-17 and IL-13 expression (*right panel*). Data represent 1 representative experiment of 2 independent experiments.

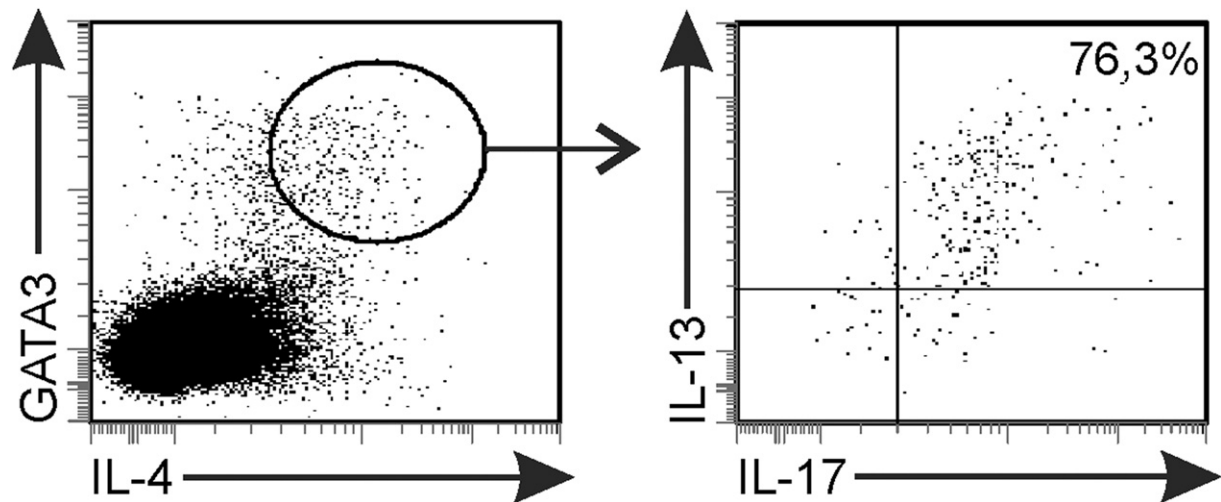


FIG E6. OVA-DCs convert lung effector $CD4^+$ T cells into $T_H17^+T_H2^+$ cells that express GATA-3 *in vitro*. CD45.2 mice were injected with OVA-CD45.1 DCs intravenously at days 0 and 12 and killed on day 15. OVA-CD45.1 DCs were cocultured with lung effector $CD4^+$ T cells. IL-13 and IL-17 expression among IL- 4^+CD4^+ T cells that expressed GATA-3. Data are from 1 representative experiment of 2 independent experiments.