Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function

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Airway DCs play a crucial role in the pathogenesis of allergic asthma, and interfering with their function could constitute a novel form of therapy. The sphingosine 1–phosphate receptor agonist FTY720 is an oral immunosuppressant that retains lymphocytes in lymph nodes and spleen, thus preventing lymphocyte migration to inflammatory sites. The accompanying lymphopenia could be a serious side effect that would preclude the use of FTY720 as an antiasthmatic drug. Here we show in a murine asthma model that local application of FTY720 via inhalation prior to or during ongoing allergen challenge suppresses Th2-dependent eosinophilic airway inflammation and bronchial hyperresponsiveness without causing lymphopenia and T cell retention in the lymph nodes. Effectiveness of local treatment was achieved by inhibition of the migration of lung DCs to the mediastinal lymph nodes, which in turn inhibited the formation of allergen-specific Th2 cells in lymph nodes. Also, FTY720-treated DCs were intrinsically less potent in activating naive and effector Th2 cells due to a reduced capacity to form stable interactions with T cells and thus to form an immunological synapse. These data support the concept that targeting the function of airway DCs with locally acting drugs is a powerful new strategy in the treatment of asthma.

Introduction
DCs are powerful antigen-presenting cells with a unique capacity to stimulate naive T cells (1). In the airways, immature lung DCs are ideally placed to sample inhaled antigens (2). After they acquire antigens in the lung, DCs migrate to the draining mediastinal LNs, where they localize in the T cell-rich area, and initiate immune responses. It was recently shown that induction of T cell division and effector generation is a specific function of myeloid DCs (mDCs) whereas induction of tolerance is a specialized function of lung plasmacytoid DCs (pDCs) (3–7). Not surprisingly, DCs have also been implicated in the process of Th2 sensitization that is typical of allergic asthma (2). The mDCs have additional functions in established allergic inflammation, as their conditional removal abolishes all the cardinal features of asthma, such as Th2 cytokine-dependent airway eosinophilia, goblet cell hyperplasia, and bronchial hyperreactivity (8, 9). Therefore, drugs that interfere with DC function in the airways hold great promise for being novel antiasthmatic compounds. Preferably, these drugs should be administered via inhalation to avoid systemic immunodeficiency.

The lymphophospholipid growth factor sphingosine 1–phosphate (S1P) is predominantly generated by stimulated platelets and leukocytes and is present in micromolar concentration in serum and extracellular fluid (10, 11). In immune cells, S1P can modulate many different functions, including migration and cytokine and chemokine release (11, 12). The novel immunomodulator FTY720 is a chemical derivative of myriocin, a metabolite of the ascomycete Isaria sinclairi, and its phosphorylated metabolite FTY720-P is a structural homolog of S1P. FTY720 acts through 4 of the 5 G protein–coupled S1P receptors (S1PR1, S1PR3–5) (10, 11, 13). These receptors regulate cytoskeleton-dependent cellular processes, including maintenance of cellular morphology and leukocyte migration (10, 11, 13–15). FTY720 modulates lymphocyte trafficking in a dual manner by accelerating migration into secondary lymphoid organs and blocking the egress into the medullary sinus of LNs and efferent lymphatics, leading to an overall decrease in circulating T and B lymphocytes (lymphopenia) and inhibition of lymphocyte influx into sites of inflammation (14, 16, 17). This mechanism of action makes it highly effective in suppressing transplant rejection (18–21) and T cell–mediated inflammatory diseases (14, 16).

Recently, oral treatment with FTY720 was shown to inhibit airway inflammation induced by adoptive transfer of Th1 and Th2 cells and asthma induced by active immunization and challenge with OVA (22). However, as in all other disease models of allorejection and autoimmunity, the therapeutic effects were claimed to result from T cell sequestration in lymphoid tissues, leading to peripheral lymphopenia (13). It has, however, been shown that FTY720 can also affect other cell types involved in allergic asthma, including DCs. We and others have reported that human immature and mature monocyte–derived DCs express S1PR and that S1P and FTY720 can modulate the migration, cytokine production profile, T cell stimulatory function, and maturation of human DCs in vitro (23, 24). The aim of the present study was therefore to investigate whether local treatment with FTY720 could suppress experimental asthma, focusing particularly on how it affected lymphocyte distribution and DC function.

Nonstandard abbreviations used: Ag, antigen; BAL, bronchoalveolar lavage; BMDC, BM-derived DC; BM, bone marrow; DR, DQ, DP, MHC class II; I-OVA, i-OVA; OVA-pulsed DCs, DCs pulsed with OVA; Penh, enhanced pause; S1P, sphingosine 1–phosphate; S1PR, S1P receptor.

Conflict of interest: The authors have declared that no conflict of interest exists.

Results
Local application of FTY720 and S1P during allergen challenge suppresses the cardinal features of asthma. We investigated whether local application of FTY720 or its physiological analogue S1P occurring 30 minutes prior to each allergen challenge would influence the development of eosinophilia in already sensitized mice. In these experiments, sensitization to OVA was induced using i.p. injection of OVA in the Th2 adjuvant alum (8). Control mice were sham sensitized using PBS/alum followed by challenge with OVA aerosol. As expected, OVA-sensitized mice treated with the vehicle prior to each OVA aerosol challenge developed bronchoalveolar lavage (BAL) fluid eosinophilia and lymphocytosis accompanied by enhanced Th2 cytokine production in mediastinal LNs (Figure 1, A–F). Treatment with FTY720 (Figure 1, A, C, and G) or S1P (Figure 1, B, D, and H) prior to each allergen challenge resulted in a significant reduction of the lymphocyte and eosinophil infiltrate into the BAL compartment and in peribronchial and perivascular inflammation on lung sections. The dramatic reduction of airway inflammation in FTY720- and S1P-treated mice was accompanied by significantly reduced levels of IL-4, IL-5, and IL-13 in mediastinal LNs while the concentration levels of IFN-γ and IL-10 were not significantly changed (Figure 1, C and D, and data not shown).

When OVA-sensitized mice were first subjected to 3 OVA aerosol exposures and subsequently exposed to another 3 daily OVA or PBS aerosol challenges on days 20–22. Thirty minutes before each of these aerosol challenges, mice received an i.t. injection of control vehicle or 0.25 μg FTY720. Twenty-four hours after the last OVA or PBS exposure, BAL was performed. Labels indicate sensitization/first series of aerosol challenges/treatment/second series of aerosol challenges. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, representative of 3 independent experiments.

Figure 1
Effect of local FTY720 and S1P treatment on asthma features. Mice were sensitized by an i.p. injection of OVA/alum on days 0 and 7. On days 19–21, mice were subjected to OVA aerosol challenges. Thirty minutes before each aerosol challenge, mice received an i.t. injection of vehicle, 0.25 μg FTY720, or S1P 10⁻⁶ M. (A and B) BAL fluid was analyzed by flow cytometry. (C and D) Mediastinal LN cell suspensions were restimulated in vitro for 4 days with OVA and assayed for cytokine production using ELISA assay. (E–H) H&E staining of lung sections of mice treated or not with FTY720 and S1P. Data in E and F are from the same experiments as in A and B, respectively. (I) FTY720 inhibits the induction of BHR. Airway obstruction to increasing doses of inhaled methacholine was measured as increase in Penh values (% increase from saline) 24 hours after the last Ag exposure. Labels indicate immunization/treatment/challenge; n = 6–8 mice per group. Data are shown as mean ± SEM. (J) Sensitized BALB/c mice were first subjected to OVA aerosol challenges on days 17–19. Subsequently, mice were subjected to an additional series of 3 daily OVA or PBS aerosol challenges on days 20–22. Thirty minutes before each of these aerosol challenges, mice received an i.t. injection of control vehicle or 0.25 μg FTY720. Twenty-four hours after the last OVA or PBS exposure, BAL was performed. Labels indicate sensitization/first series of aerosol challenges/treatment/second series of aerosol challenges. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, representative of 3 independent experiments.
administration of FTY720 did not lead to measurable systemic levels of the drug, as measured by liquid chromatography–mass spectrometry (LC-MS) (21) (V. Brinkmann, Novartis Research Institute, Basel, Switzerland, unpublished observations).

One of the cardinal features of asthma is the presence of bronchial hyperresponsiveness to nonspecific stimuli, such as inhaled methacholine. As shown in Figure 1I, OVA challenge of OVA-sensitized mice induced a significant change in responsiveness to methacholine. As shown in Figure 1I, OVA challenge of OVA-sensitized mice induced a significant change in responsiveness to methacholine. As shown in Figure 1I, OVA challenge of OVA-sensitized mice induced a significant change in responsiveness to methacholine.

Effect of local FTY720 treatment on number of lymphocytes in peripheral blood (A), peripheral LNs (B) and lung-draining LNs (C). Single-cell suspensions from blood, peripheral LNs, and lung-draining LNs were made and stained for the presence of CD4+ or CD8+ T cells and CD19+ B cells. Labels indicate sensitization/treatment/challenge. Treatment consisted of 0.25 μg of FTY720 or vehicle. Data were calculated as percentage of nonerythrocytes (A) or as absolute cell numbers (B and C). MLN, mediastinal LN; PLN, peripheral LN. Data indicate mean ± SEM. **P < 0.01; ***P < 0.001, representative of 3 independent experiments.

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DC migration (7). The number of MHCII⁺CD11c⁺ DCs carrying fluorescent FITC-OVA cargo was enumerated in the mediastinal LNs 24 hours after instillation, and as shown in Figure 5A, concomitant treatment with 0.25 μg FTY720 significantly inhibited the migration of lung DCs to the LNs. At the same time, significantly more FITC⁺ DCs were retained in the lungs after FTY720 treatment (Figure 5B). These data also show that FTY720 is not toxic to lung DCs but rather retains them in the lung. On an individual cell basis, there was no difference in the intensity of FITC-OVA staining in lung or LN DCs exposed to FTY720 or not, illustrating that the drug did not interfere with antigen (Ag) uptake. In vitro experiments revealed further that FTY720-treated DCs were still able to migrate in response to the CCR7 agonist chemokine CCL19 but no longer migrated in response to S1P (Supplemental Figure 1; available online with this article; doi:10.1172/JCI28295DS1), suggesting that a loss of chemotactic responsiveness to LN-expressed S1P was the primary cause of reduced migration of DCs to the draining nodes.

Effect of FTY720 on Th2 priming induced by lung mDCs. Increased migration of DCs to the draining LNs is an integral part of both the primary and secondary immune response to inhaled allergens, and it has been shown that this migration is essential for generating Th2 effector function from resting T cells in LNs (9, 27–29). To find out whether local FTY720 treatment can also suppress development of new Th2 effector cells in response to inhaled OVA, we made use of a model in which tolerogenic pDCs are first removed from the system through treatment with a pDC-depleting Ab, GR1-Ab (3). As previously reported, when 800 μg of OVA was given i.t. to naive mice with normal numbers of pDCs (isotype control treatment), this did not lead to Th2 priming, and a subsequent allergen challenge 10 days later did not lead to airway eosinophilia (Figure 6). However, when pDCs were depleted with anti-GR1, i.t. administration of OVA led to Th2 priming, as exemplified by strong airway eosinophilia and lymphocytosis in the BAL fluid and eosinophilia and goblet cell hyperplasia in the lung upon allergen challenge to the lung (Figure 6, A and B).

When the first i.t. OVA-priming dose was admixed with FTY720 in pDC-depleted mice, the number of eosinophils and lymphocytes were strongly reduced in BAL fluid (Figure 6A) and lung tissues (Figure 6B) compared with those in mice receiving vehicle admixed OVA. In addition, the generation of OVA-specific effector Th2 cells in the mediastinal nodes in pDC-depleted mice was also suppressed by FTY720 (Figure 6C).

Effect of in vitro FTY720 treatment on potential of mDCs to induce Th2 development in vivo. In the experiments described above, the inhibitory effects of FTY720 on Th2 sensitization could also be due to influences on structural airway cells. We therefore performed experiments in which mDCs were treated ex vivo with FTY720 prior to i.t. transfer in vivo. As the number of DCs obtained from the lungs of mice was too small to perform large adoptive transfer experiments, we decided to use BM-derived DCs (BMDCs). Similarly to the in vivo effects on lung DCs, the maturation state (MHCII, CD40, CD80, and CD86) of OVA-pulsed BMDCs was not affected by prior overnight incubation with FTY720 (data not shown). As previously reported, adoptive i.t. transfer of OVA-pulsed mDCs leads to Th2 priming, and eosinophilic airway inflammation subsequently develops upon OVA aerosol challenge 10 days later (6). As expected, in mice receiving unpulsed DCs, only a few inflammatory cells were observed in the BAL fluid (Figure 7, A and B). Mice immunized with OVA-pulsed DCs demonstrated a strong cellular recruitment of lymphocytes and eosinophils in the BAL fluid (Figure 7A) and peribronchial and perivascular area (Figure 7B) while goblet cell hyperplasia was induced. The pretreatment of OVA-pulsed DCs (OVA-DCs) with FTY720 ex vivo significantly inhibited in a dose dependent manner (data not shown) the development of these salient features of asthma. This was accompanied by a significant decrease in the levels of IL-4, IL-5, and IL-13 in the mediastinal LNs even though the concentration of IL-10 was not

**Figure 3**
Expression of S1PRs on lung DCs. RT-PCR analysis of S1PR (S1PR1–5) expression was performed on highly purified, freshly isolated lung DCs. Far left and far right columns show 100-bp DNA ladder.

**Figure 4**
Effect of local FTY720 treatment on the number of DCs in peripheral blood (A), peripheral LNs (B), and lung-draining LNs (C). Single-cell suspensions from blood, peripheral, and mediastinal LNs of PBS/vehicle/OVA- or OVA/vehicle/OVA- or OVA/FTY720/OVA-treated mice were prepared. The cells were stained with anti-CD11c and anti-MHCII and analyzed by flow cytometry. Autofluorescent and PI-positive dead cells were excluded from the gate. Data were calculated as absolute number of cells. Data indicate mean ± SEM. **P < 0.01; ***P < 0.001, representative experiments out of 3.
A decrease in the levels of IFN-γ may also result from an inherent effect of FTY720 on the stimulation of mDCs with 10^6 M S1P driven Th2 sensitization may be due in part to inhibition of DC migration to mediastinal LNs but was not due to the reduced capacity of FTY720-treated DCs to process whole OVA protein, as OVA-peptide–pulsed DCs also formed less stable interactions when treated with FTY720 (Figure 9A).

Figure 5
Effect of local FTY720 administration on lung DC migration to the lung-draining LNs. (A) On day 0, mice were instilled i.t. with 10 mg/ml FITC-OVA with or without 0.25 μg FTY720. Twenty-four hours later, the presence of FITC+ migrating DCs in thoracic draining LNs was analyzed by flow cytometry. (B) Effect of FTY720 on lung DCs. Twenty-four hours after installation of FITC-OVA/vehicle, lungs were enzymatically digested and stained for the presence of FITC–MHCII+CD11c+ DCs. Experiments were repeated 3 times with similar results; n = 4 mice per group. Data indicate mean ± SEM. *P < 0.05; **P < 0.01.

Discussion
Th2 lymphocytes play a crucial role in allergic asthma, and strategies aimed at interfering with the function of these cells or individual cytokines (anti–IL-5, soluble IL-4 receptor) have been proposed as novel forms of therapy (32). Recently, the immunomodulator FTY720 has been shown to be effective as an oral drug for the treatment of experimental asthma, induced by adoptive transfer of effector Th2 cells (22). In addition, numerous other Th1-mediated experimental disease models of graft rejection, type 1 diabetes mellitus, autoimmunity, and arthritis have been successfully treated using FTY720, and this drug is currently employed in phase III clinical trials for prevention of allograft rejection (18, 20). In all of these models, FTY720 has been administered systemically where its major mechanism of action is the inhibition of lymphocyte egress from the LN medulla, resulting in peripheral lymphopenia (17, 19, 21). S1PR agonists cause sequestration of lymphocytes in secondary lymphoid tissues by acting as functional antagonists that downregulate S1PR1s on lymphocytes or by preventing entry of medullary lymphocytes into the effenter lymphatics for recirculation (17). Although FTY720 does not impair the activation, expansion, and memory function of lymphocytes (33), the occurrence of peripheral lymphopenia and, as a consequence, the reduced recruitment of effector lymphocytes to sites of inflammation might constitute a form of immunodeficiency, with its inherent risk of developing opportunistic infections, which is undesirable for the treatment of non–life threatening diseases, such as allergic asthma. To avoid this systemic immunodeficiency, we tested to determine whether local i.t. treatment with FTY720 was similarly able to suppress the cardinal features of asthma. Local FTY720 treatment given prior to each allergen challenge in sensitized mice was able to inhibit the salient pathologies of asthma, including eosinophilic inflamma-
Local administration of FTY720 prevents asthma development driven by endogenous mDCs. On day 0, mice received an i.t. injection of OVA in the presence or absence of FTY720. From days –1 to 2, mice were injected i.p. with anti-GR1 Abs to deplete tolerogenic pDCs or isotype control Abs. Ten days later, mice were subjected to 3 OVA aerosol challenges. (A) BAL fluid was analyzed by flow cytometry. (B) H&E staining of lung sections. (C) MLN cells restimulated in vitro for 4 days with OVA and assayed by ELISA. Data indicate mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, representative of 2 independent experiments.

DCs are crucial in both the initiation and maintenance phase of allergic asthma and therefore constitute ideal targets for the development of novel antiasthmatic drugs (2). As a proof of concept, selective depletion of DCs during allergen challenge abolishes all the cardinal features of asthma, including bronchial hyperreactivity (30). DCs are crucial because they can locally activate Th2 effector cells in the airway wall by providing chemotactic cues for Th2 cells (CCL17 and CCL22) and by delivering MHC and costimulatory signals (4, 35–37). Additionally, at times of allergen challenge, DCs migrate from the site of allergic inflammation to mediastinal LN to stimulate new rounds of division in recirculating central memory cells or from naive T cells, thus feeding the inflammatory response with new waves of effector cells (2, 27–29). As FTY720 and S1P suppressed inflammation without altering lymphocyte distribution, we studied the effect of FTY720 and S1P on lung DCs and found that freshly isolated lung DCs express all S1PR subtypes, consistent with prior reports on cultured DCs (23, 26). One of the striking observations we made after local FTY720 treatment was a reduction in the number of mDCs in mediastinal LNs of allergen-challenged mice. Although, again, a reduction in lung inflammation might be responsible for reduced input of immigrating lung-derived DCs in LNs, we also observed a reduction in DC migration to LNs in naive mice when FTY720 was administered together with fluorescently labeled OVA, concomitant with an accumulation of DCs in the lung compartment. The same effect of systemically administered FTY720 and S1P agonists was seen on DC migration from the skin following FITC skin painting (26, 38).

We and others have shown that S1P agonists and FTY720 alter the regulation of actin-dependent cytoskeletal processes in DCs by downstream signaling of different S1PRs through small Rho family GTPases, such as Rho and Rac/Cdc42 (23, 38). It was shown that a correct balance and possibly cyclic activity of Rho and Rac/Cdc42 are essential for DC migration to occur and that this process might be inhibited by FTY720 (38). However, this explanation seemed less likely as we performed in vitro chemotactic assays. DC migration from the periphery of the lung to the mediastinal nodes is CCR7 dependent (39). It has been shown by Lan et al. that FTY720 treatment of DCs impairs CCR7-dependent chemotactic migration to CCL19 across endothelial cells in vitro (26) whereas Czeloth et al. showed that migration to the other CCR7 ligand, CCL21, across Transwell inserts was unaffected (38). In our experiments, we found that FTY720 treatment of DCs did not affect migration toward CCR7 ligands in vitro whereas, clearly, responsiveness to the chemotactic signal S1P was reduced. We therefore favor the idea that FTY720 specifically suppresses migration of DCs by downregulation of the S1PR1 and not by interference with the actin cytoskeleton.

Inhalation of low-endotoxin OVA is a normally tolerogenic event in which pDCs inhibit the potential of mDCs to prime for effector
Th2 cells (3–5). By depleting pDCs, this tolerogenic response is turned into robust Th2 priming by mDCs. In this model, FTY720 completely abolished the development of Th2 effector cells, and consequently, asthma did not develop upon repeated OVA challenge. There may be several reasons for the lack of Th2 priming. First, S1P agonists have been shown to alter the function and stability of bronchial epithelial tight junctions (40). These tightened junctions could be a barrier to the uptake of inhaled OVA, thus leading to a lack of Th2 priming. To exclude this possibility, we also incubated mDCs with FTY720 or S1P prior to adoptive transfer to naïve mice. This pretreatment of DCs significantly reduced their potential to induce Th2 priming, and consequently, asthmatic inflammation did not develop. Therefore, the effects of FTY720 are most consistent with an autonomous defect on DCs.

Second, FTY720 inhibited the migration of lung-derived DCs to LNs, thus inhibiting proper T cell priming. Priming of recirculating naïve T cells or reactivation of central memory T cells in LNs depends on input of DCs from the periphery of the lung in a process that is CCR7 dependent (39). However, inhibition by FTY720 of DC-mediated FITC-OVA transport was not absolute, and the DCs that escape FTY720 inhibition might still induce T cell activation. This indeed was the case when we followed primary T cell activation in a cohort of adoptively transferred DO11.10 T cells in vivo (data not shown). Even in FTY720-treated mice, OVA inhalation still led to T cell divisions in mediastinal nodes, although the strength of the T cell response was reduced. We therefore favor the third possibility that FTY720- or S1P-treated DCs are also defective in priming naive and memory T cells due to an intrinsic defect in T cell stimulatory capacity. Consistent with this, we cultured OVA-pulsed BMDCs with OVA-specific T cells from naïve mice and observed a strong and dose-dependent reduction in proliferation and production of both Th1 and Th2 effector cytokines when DCs were preincubated with FTY720. In addition, FTY720 treatment of DCs inhibited their potential to stimulate primed effector Th2 cells generated under polarized Th2 conditions in vitro. Similar effects of FTY720 and S1P were noticed on the immunostimulatory capacity of human monocyte-derived DCs matured using LPS although in the human setting, Th1 cytokines were preferentially inhibited while Th2 priming was essentially weak but unaffected (23, 24). Our results showed that local FTY720 treatment inhibits IL-12 production in lung DCs, a finding similar to that in human data.

FTY720 might modify the potential of DCs to stimulate T cells in several ways. The induction and maintenance of effector cytokine production in T cells in the lungs depends upon an adequate antigen-processing function and maturation of lung DCs (41). Surprisingly, neither FTY720 nor S1P altered the expression of CD40, CD80, CD83, CD86, programmed death ligand-1 (PDL-1), PDL-2, and MHCII in immature or mature BMDCs (data not shown). Moreover, local FTY720 treatment did not alter the maturation of lung and LN DCs. These data are in accordance with studies in which S1P agonism did not affect maturation of murine and human DCs (23, 24, 26). FTY720 treatment of DCs could also interfere with their potential to form stable and long-lasting interactions with T cells in the LNs or lung parenchyma. The small Rho family GTPases (such as Rac1 and Rac2) are modulated by FTY720, and expression of these factors in DCs has been shown to be essential for the occurrence of DC–T cell interaction (31). In accordance, FTY720 treatment of DCs indeed abolished the formation of long-lasting DC–T cell interactions typical of an immunological synapse that is critical for induction of cellular immunity (31). Whether local treatment with FTY720 would also reduce the formation of an immunological synapse between recirculating T cells and lung DCs in the airways or lung parenchyma remains to be shown.

In conclusion, the present study demonstrates for what we believe is the first time that the local administration of FTY720 inhibits...
Th2-mediated cardinal features of asthma by altering the function of lung DCs without causing any systemic lymphopenia. These findings could pave the way for a feasibility study in which this compound or a more selective S1PR agonist is administered via aerosol to patients with asthma. This paper validates the concept that targeting airway DC function is a powerful method to treat asthma.

Methods

Mice. BALB/c and C57BL/6 mice (6–8 weeks old) were purchased from Harlan. OVA-TCR Tg mice (DO11.10 BALB/c and OTII C57BL/6) were bred at the Erasmus Medical Center. All experiments were subject to review by an independent Animal Ethics Committee that approved all experimentation (Stichting DEC Consult, Utrecht, The Netherlands).

A mouse model of asthma induced by systemic i.p. sensitization. BALB/c mice (n = 6–8 per group) were sensitized to OVA by i.p. injection of OVA/Alum (10 μg OVA grade V adsorbed to 1 mg aluminum hydroxide; Sigma-Aldrich) on days 0 and 7 and were subjected to OVA aerosol challenges (grade III) on days 17–19; aerosol challenges were dispensed from a jet nebulizer delivering 1% OVA in PBS for 30 minutes. Thirty minutes before each OVA exposure, mice were anesthetized using Avertin (Sigma-Aldrich) and received an i.t. injection of control vehicle, 0.25 μg FTY720, or of 10⁻⁶ M S1P in a volume of 80 μl. Twenty-four hours after the last OVA exposure, BAL was performed and lungs and LNs were resected and digested using collagenase/DNase, as described (7, 27).

A mouse model of asthma induced by endogenous myeloid airway DCs. In order to study the effect of FTY720 on the function of endogenous airway mDCs, mice were first depleted of tolerogenic pDCs by 3 i.p. injections of the pDC-depleting antibody GR1 (RB6-8C5) on days –1, 0, and 1 as described (3). On day 0, 800 μg of OVA (LPS low; Worthington Biochemical Corp.) was injected i.t., admixed with 0.25 μg of FTY720 or vehicle. After a washout period of 10 days, mice were subjected to 3 OVA aerosol challenges of 30 minutes on each of 3 consecutive days.

A mouse model of asthma induced by adoptive transfer of BMDCs. We have developed a model in which sensitization to inhaled OVA is induced by i.t. injection of OVA-pulsed BM-derived mDCs (6). DCs were prepared as previously described, by growing BM cells in medium containing recombinant mouse GM-CSF. At day 9 of culture, cells were pulsed overnight with 1 μg of OVA or S1PR agonists, and resuspended in PBS at a concentration of 1.25 × 10⁶ cells/ml.

For in vivo experiments, BALB/c mice were anesthetized on day 0 with Avertin (2% v/v in PBS), and 1 × 10⁶ vehicle DCs, OVA-DCs, FTY720-DCs or S1P-DCs were instilled through the opening vocal cords, as described (6). On days 10–12, mice were exposed to a 30-minute OVA aerosol challenge. Mice were sacrificed 24 hours after the last aerosol challenge.

Flow cytometry and sorting. BALC were stained for 30 minutes with anti-I-Ad/I-Ed FITC (macrophages/DCs), anti-CCR3 PE (eosinophils), anti-CD3 and anti-CD19 FITC (lymphocytes) (eBioscience), and anti-CD11c APC (macrophages/DCs), with PE-labeled anti-CD40, anti-CD80, anti-CD83, and anti-CD86, and with APC-labeled anti-CD11c. For in vivo experiments, BALB/c mice were anesthetized on day 0 with Avertin (2% v/v in PBS), and 1 × 10⁶ vehicle DCs, OVA-DCs, FTY720-DCs or S1P-DCs were instilled through the opening vocal cords, as described (6). On days 10–12, mice were exposed to a 30-minute OVA aerosol challenge. Mice were sacrificed 24 hours after the last aerosol challenge.

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For determination of systemic lymphocyte distribution, heparinized blood samples (200 μl) were lysed using hypotonic ammonium chloride buffer and stained for T cell subsets (FITC-anti-CD3, PE-anti-CD4, Cyrome-anti CD8), B cells (PE-anti-CD19) or DCs (FITC-anti-MHCII, APC-anti-CD11c, PE-anti-CD11b). Peripheral (axillary and inguinal) and mediastinal LN cells were stained with the same combination. Absolute cell number was calculated by multiplying the total leukocyte number by the percentage (%) of each subset.

For analysis of DC maturation, BM, lung, or LN cell suspensions were stained with FITC-labeled anti–I-Ad, I-Ed, with PE-labeled anti-CD40, anti-CD80, anti-CD83, and anti-CD86, and with APC-labeled anti-CD11c antibodies. For sorting of DCs from lung suspensions, nonautofluorescent CD11c⁺, MHCI⁺CCR3⁺CD3⁺CD19⁺ cells were sorted using a FacsARIA flow cytometer (BD) as described (42).

To address migration of lung DCs, 80 μl of FITC-OVA (10 mg/ml) with or without 0.25 μg FTY720 or control DMSO vehicle was administered i.t. through the opening vocal cords using an 18-gauge polyurethane catheter connected to the outlet of a micropipette as previously described (7). At
24–36 hours after injection, migrating DCs were enumerated in mediastinal LNs as CD11c+MHCII+ cells carrying FITC+ material.

In all experiments, dead cells were excluded from analysis using propidium iodide. Analysis was performed on a FACSCalibur flow cytometer (BD), using CellQuest (version 3.3; BD) and FlowJo software (version 6.4.7; Tree Star Inc.).

Expression of S1PRs on lung DCs. Lung DCs were sorted using flow cytometry, and total RNA was extracted from the cells using TRIzol Reagent (Invitrogen). To obtain cDNA, 5 μg of total RNA was primed with oligo-8 dT primers (Hermann Synthetische Biomoleküle) and reverse transcribed with StrataScript Reverse Transcriptase (Stratagene). Primers for the different murine S1PRs were designed to fit published sequence data (43). PCR was performed using 10 μl of IQ Supermix (Bio-Rad), 7 μl H2O, 1 μl of each primer (final concentration 0.5 μM each), and 1 μl cDNA. Amplification conditions were as follows: 12 minutes initial denaturation at 95°C followed by 45 cycles of denaturation (94°C) for 30 seconds, annealing (57°C for S1PR1–4, 62°C S1PR5) for 30 seconds, and amplification (72°C) for 45 seconds. PCR products were separated by electrophoresis on a 2.5% agarose gel and visualized by ethidium bromide staining.

Determination of BHR. Twenty-four hours after the last OVA aerosol challenge, nonspecific airway responsiveness was measured by exposing awake mice to aerosolized PBS to set a baseline value, followed by increasing concentrations of aerosolized methacholine (1.5625, 3.125, 6.25, 12.5, and 25 mg/ml in PBS for 3 minutes; Sigma-Aldrich) using ultrasonic nebulizers. Penh values were measured for 3 minutes after each methacholine aerosol using a whole-body plethysmograph (Buxco). The average Penh values were expressed for each methacholine concentration as the percentage increase over baseline Penh values (44).

Activation of OVA-specific naive and effector T cells by mDCs. Naïve CD4+ T cells (1 × 106) were purified from unmanipulated DO11.10 TCR Tg (H-2b) mice and were cocultured with BMDCs (1 × 105), which were pretreated with the indicated concentrations of FTY720, S1P, or vehicle alone and 10 μg/ml OVA protein in round-bottom 96-well tissue culture plates. In separate experiments, naïve DO11.10 T cells were first differentiated for 7 days into effector Th2 cells in the presence of IL-4, anti–IFN-γ, and anti-IL-12 as previously described (30). After washing, these effector Th2 cells were stimulated with DCs as for naïve T cells. After 4 days, supernatants were harvested and analyzed for IL-4, IL-5, IL-12, and IL-10 cytokines by ELISA. In separate wells, proliferation was measured by pulsing with [3H]thymidine (1 μCi/well) for the last 16 hours of culture, followed by measurement of uptake in a beta counter (TopCount; Packard; GMI). Results are given as mean cpm ± SEM of triplicate cultures.

To visualize dynamic interactions between DCs and specific T cells, OVA-specific T cells obtained from OTII TCR Tg (H-2b) mice were cocultured with unpulsed BMDCs, OVA protein–pulsed BMDCs (100 μg/ml), or OVA peptide–pulsed BMDCs (1 μg/ml) treated or not with 0.25 μg FTY720 overnight in an imaging chamber at 37°C in an atmosphere of 5% CO2. Prior to incubation, DCs were stained with an FITC-labeled antibody to I-A^b (M5/114), and T cells were stained with biotin-tagged TCR αβ antibodies, followed by Qdot 655 streptavidin conjugates (Invitrogen). Interactions were visualized with an LSM 510 confocal microscope (Zeiss) equipped with 488-nm and 633-nm lasers. Time-lapse images were collected every 20 seconds for 40 minutes. Interactions were quantified using Imaris software (version 5.0; Bitplane AG).

Cytokine measurements. To measure cytokine levels, mediastinal LN cells were plated in round-bottom 96-well plates (1 × 105 cells/ml) and restimulated with OVA (10 μg/ml) for 4 days. The presence of IL-4, IL-5, IL-10, IL-13, and IFN-γ was assayed on supernatants by ELISA (BD).

Statistics. For all experiments, the statistical significance of differences between samples was calculated using the Mann-Whitney U test for unpaired data. Differences were considered significant at P < 0.05.
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