

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Topical treatment targeting sphingosine-1-phosphate and sphingosine lyase abrogates experimental allergic rhinitis in a murine model

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Keywords

allergic rhinitis; dendritic cell; eosinophils; sphingosine lyase; sphingosine-1-P.

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Abstract

Background: Sphingosine-1-phosphate (S1P) plays a crucial role in homeostasis of the immune system by regulating lymphocyte recirculation and inflammatory cell recruitment. The levels of S1P are tightly controlled through regulated production and controlled breakdown by sphingosine-lyase (SL). The S1P analogue FTY720 has been developed as an immunosuppressant in transplantation and tested as a treatment for various inflammatory diseases. FTY720 exploits S1P biology by acting as a S1P1 and S1P 3 agonist and by inhibiting S1P breakdown by SL.

Objective: Here, we investigate interfering S1P in allergic rhinitis (AR) and its way of action.

Methods: Allergic rhinitis was induced by sensitizing mice to ovalbumin (OVA) and challenging the nose with OVA allergen. At the time of allergen challenge, mice received topical intranasal treatment with FTY720. To address the relative contribution of SL inhibition in mediating its effects, some mice were treated with the SL inhibitor 2-acetyl-4-tetrahydroxybutyl (THI).

Results: FTY720 treatment resulted in significantly fewer eosinophils, mast cells and dendritic cells in the nasal mucosa of AR animals, compared with diluent treatment. Levels of IL-4, IL-5, IL-10 and IL-13 produced by lymph node cells fell significantly in FTY720-treated animals. Moreover, FTY720 proved potent enough to suppress inflammation in a model of persistent AR. *In vitro* and *in vivo* experiments indicate that FTY720 impaired Th2 differentiation and proliferation important in driving eosinophilia and induced apoptosis in mast cells.

Conclusion: Our results indicate that interfering with S1P metabolism is a powerful and feasible strategy to develop new topical agents that suppress AR.

Allergic rhinitis (AR) patients suffer from nasal symptoms caused by allergic inflammation rich in eosinophils and mast cells and controlled by Th2 type lymphocytes that produce IL-4, IL-5 and IL-13. Mast cell activation, however, also leads to more delayed and *de-novo* cytokine and chemokine production thus leading to the activation and recruitment of more inflammatory cells (1, 2). Dendritic cells and Th2

lymphocytes play a predominant role in this late allergic response and chronic allergic inflammation of the nose (3). Not surprisingly, depletion of mast cells, DCs and Th2 cells has been shown to interfere with chronic allergic inflammation typical of AR (4–6).

In search of new compounds that can broadly suppress allergic inflammation, we and others have found that interfering with sphingolipid metabolism has the potential to target upstream events in the inflammatory cascade like antigen presentation by DCs, lymphocyte recirculation and inflammatory cell recruitment (7–9). Sphingosine-1-phosphate (S1P) controls cell migration and lymphocyte egress from lymph nodes

Abbreviations

AR, allergic rhinitis; DCs, dendritic cells; OVA, ovalbumin; PBS, phosphate buffered salt.

(LN) and thymus (10). S1P lyase is known to be an important S1P degrading enzyme and is available in tissues at high concentrations. Due to the rapid breakdown of S1P in tissues, a concentration gradient is established between tissue and lymph vessels, allowing egress (10). A component of caramel III colorant, 2 acetyl-4-tetrahydroxybutylimidazole (THI), is a strong inhibitor of S1P lyase (10) that leads to increased concentrations of S1P in lymphoid tissue, lymphocyte recirculation shutdown and lymphopenia when given systemically.

In this study, we found that topical treatment with FTY720 reduces the features of AR by interfering with DC recruitment, Th2 lymphocyte polarization and survival of mast cells.

Material and methods

For material and methods, see Data S1.

Results

Local application of sphingosine-1-P analogue FTY720 abrogates the hallmarks of AR

Previously, we described an ovalbumin (OVA)-driven mouse model for AR (outlined in Fig. 1A) with the characteristic

features of eosinophilic inflammation in nasal mucosal tissue and elevated levels of Th2 cytokines in cervical LN (3). Using this model, we studied the effect of topical FTY720 treatment on key features of AR (Fig. 1B). FTY720 or PBS was administered with each OVA allergen challenge (FTY with OVA in 20 μ l PBS). When challenged with OVA-PBS, OVA-sensitized mice developed strong eosinophilic airway inflammation in the nasal mucosa, compared with mice challenged with PBS. When increasing doses of FTY720 were added to the OVA challenge to determine a dose-response curve (Fig. 1C), the dose of 6 μ g FTY720 proved to be the most effective (P , 0.05 Mann-Whitney U -test) reducing eosinophilic nasal inflammation in the epithelium and in the lamina propria and had no systemic side effects like blood lymphopenia. The highest concentration of FTY720 did not inhibit the response to the same extent as the middle concentration, suggesting a bell shaped dose-response curve.

Given the treatment effect and lack of systemic side effects, a dose of 6 μ g FTY given topically with each OVA challenge was therefore used in all other *in vivo* experiments. The significant reduction in eosinophilic inflammation was observed in the lamina propria (P < 0.05 Mann-Whitney U -test) when AR animals were treated with FTY720 (Fig. 1D).

The presence of eosinophils is an indirect proof that a Th2 lymphocyte response to intranasally applied OVA antigen

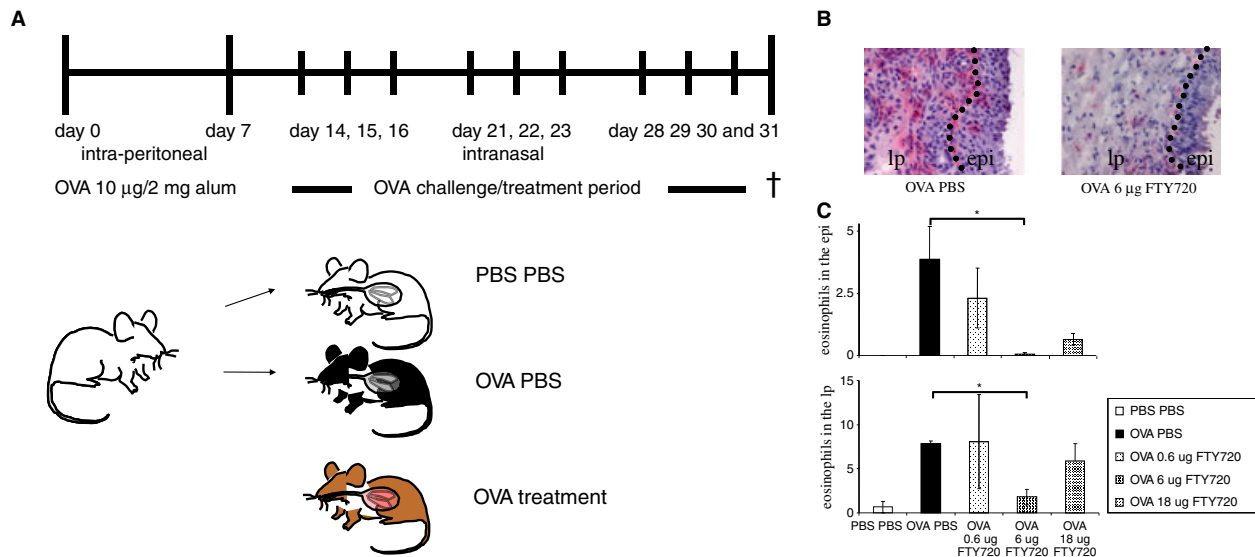


Figure 1 FTY720 treatment reduced airway inflammation in the nasal mucosa of allergic rhinitis (AR) mice. Allergic rhinitis was induced by giving ovalbumin (OVA)/alum on day 0 and day 7 followed by challenges with OVA (AR) or PBS as a control (PBS) intranasal applications on days 14–16; 21–23; 28–30. FTY treatment was given together with OVA. On day 31, mice were killed and sampled. (A) Animal treatment time schedule. (B) Representative photograph of Murine eosinophils, identified as cells with a ring-shaped or a bi-lobular nucleus (blue), and immunoreactive for MBP (red). (C) The number of eosinophils in the epithelium and the number of eosinophils in the lamina propria. Dose-response curve of FTY720 containing 0, 0.6, 6 or 18 μ g of FTY720 (respectively) in total. Data are presented as the mean $\pm$ SEM. * P < 0.05; n = 6 animals per group.

(D) Quantification of eosinophil numbers in the epithelium and lamina propria of the nasal mucosa (white bars PBS saline, black bars OVA saline and gray bars OVA FTY720). (E) Cervical lymph nodes (LN) cell suspensions were restimulated *in vitro* for 4 days with OVA and assayed for IL-4, IL-5, IL-10, IL-13 and IFN γ production using ELISA assay. (F) Quantitative RT-PCR on nasal mucosa tissue. Relative expression (ubiquitin-normalized) of IL-5, IL-13, IL-10 and FoxP3 mRNA. Significantly higher levels of expression were observed for IL-5 in OVA-PBS compared with PBS PBS or with OVA FTY-treated animals. No differences were observed in levels of IL-13, IL-10 and FoxP3. (G) Quantification of the number of CD11c-positive cells in the epithelium and lamina propria of the nasal mucosa. Data are shown as mean $\pm$ SEM. * P < 0.05; n = 5 animals per group.

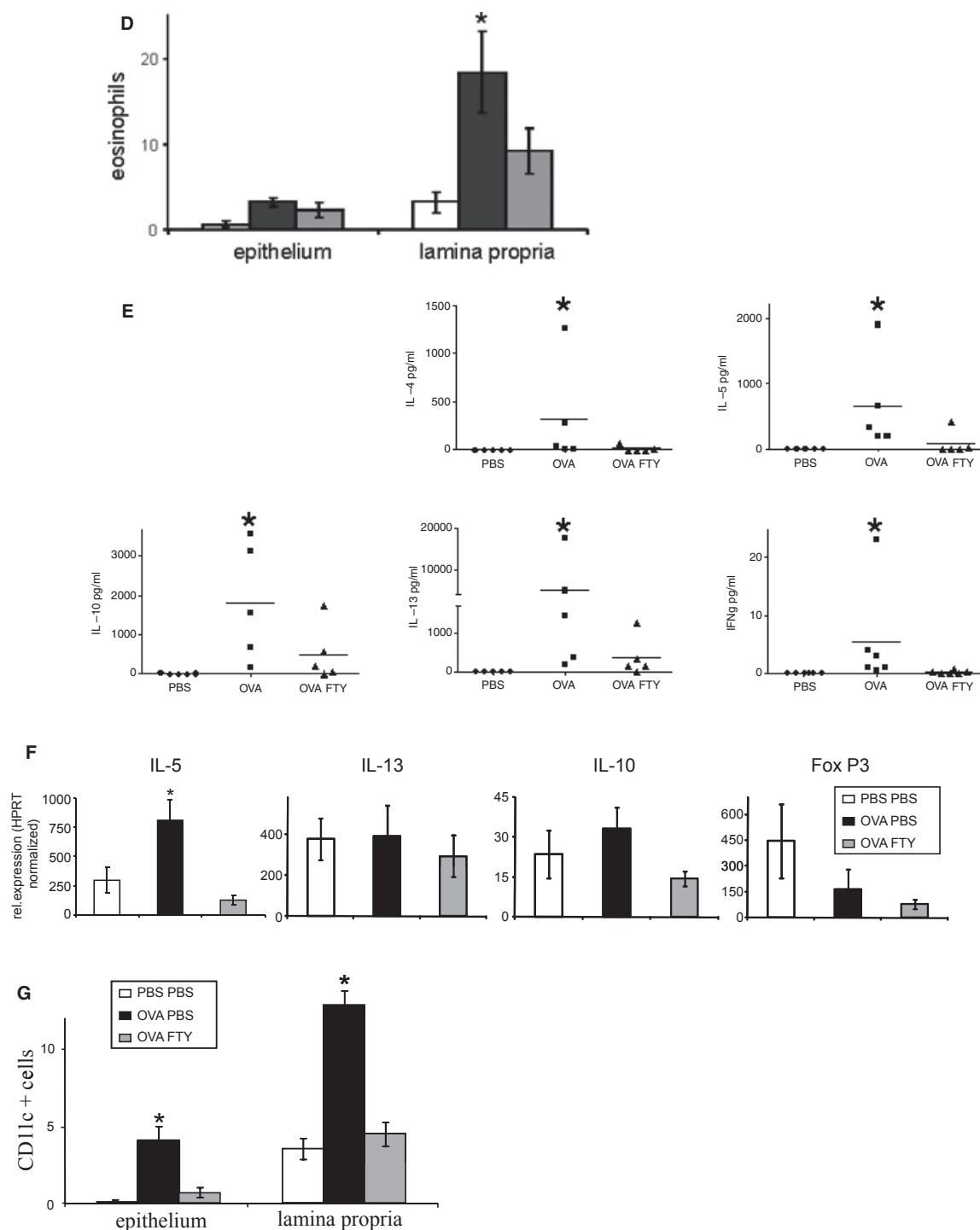


Figure 1 (continued).

was induced, but airway eosinophilia could also be induced through the actions of innate lymphoid cells producing IL-5 and/or IL-13 (11).

We therefore tested whether FTY treatment reduces adaptive Th2 immunity to OVA. T-lymphocytes obtained from

the cervical LN produced fewer Th2 cytokines (IL-4, IL-5, IL-10, IL-13) but also fewer Th1 cytokines (IFN γ) in AR animals treated with FTY720 (Fig. 1E). To address the effect of FTY720 on adaptive Th2 response more directly, we measured the levels of mRNA in the nasal mucosa tissue. Nasal

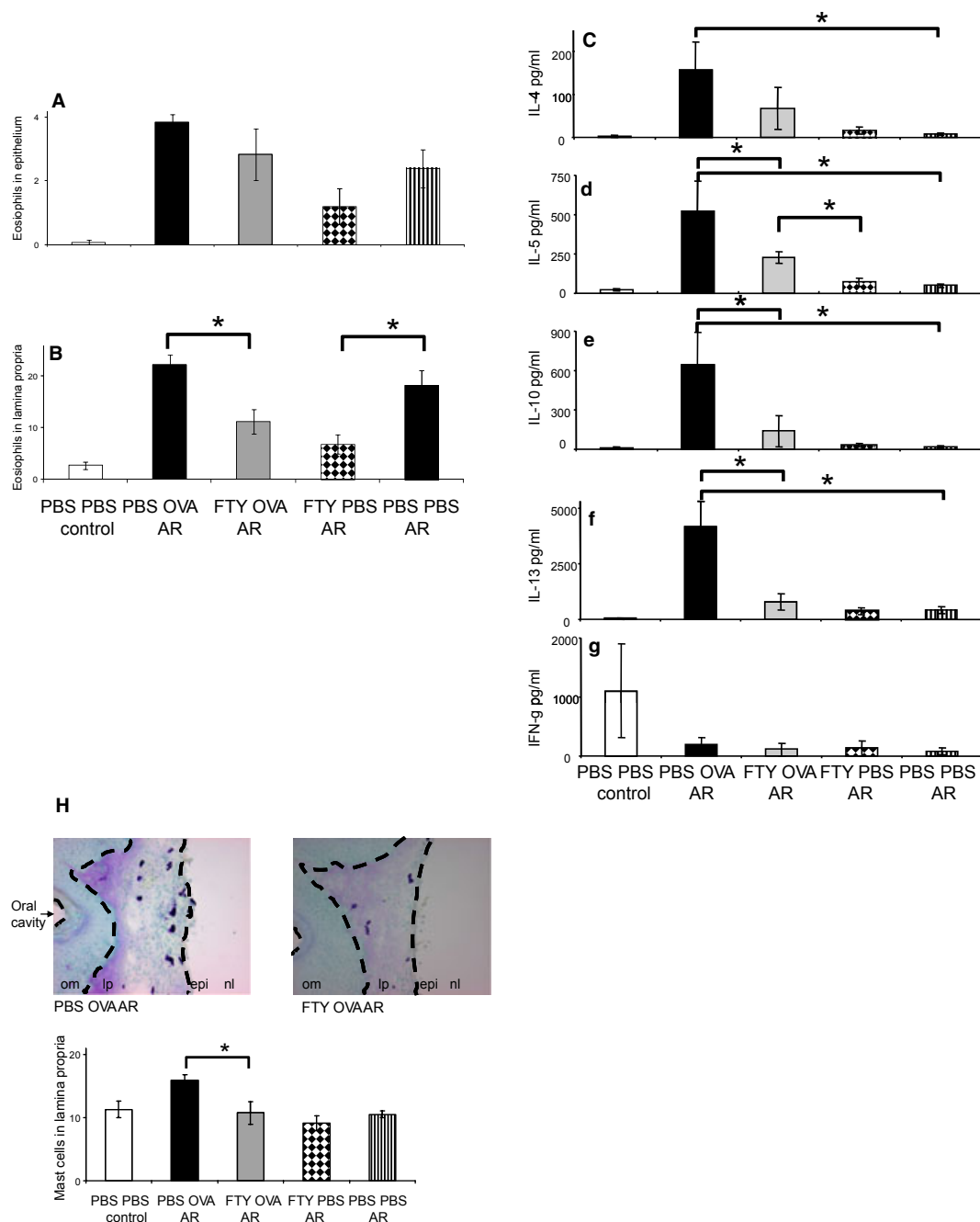


Figure 2 FTY720 treatment reduces eosinophilic inflammation during ongoing inflammation and resolution of allergic inflammation. Allergic rhinitis (AR) was induced by giving ovalbumin (OVA)/alum on day 0 and day 7 followed by challenges with OVA or PBS via intranasal applications on days 14–16, 21–23, 28–30. The animals were then challenged again on three successive days by instilling a droplet of 10 μ l PBS (PBS PBS control) or OVA (1 mg/ml) (PBS OVA AR) or OVA plus FTY 3 μ g/10 μ l treatment (FTY OVA AR) or PBS plus FTY (FTY PBS AR) or PBS (PBS PBS AR) in each nostril with a micropipettor. Twenty-four hours after the last application, mice were killed and sampled. (A) Quantification of eosinophil num-

bers in the epithelium and (B) lamina propria. (C–G) Cervical lymph nodes (LN) cell suspensions were restimulated *in vitro* for 4 days with OVA and assayed for IL-4, IL-5, IL-10, IL-13 and IFN γ production using ELISA assay. Representative photographs (H) from a cross-section of the palate. From left to right, we see the mouth (oral) cavity, palate oral mucosa (om), lamina propria (lp) including toluidine blue stained cells, on one cell layer thick epithelium (epi) including some dispersed laying epithelial cells, nasal lumen (nl). Toluidine blue-positive cells were enumerated in the lamina propria. (H) Data are presented as the mean $\pm$ SEM. * P < 0.05; n = 5 animals per group.

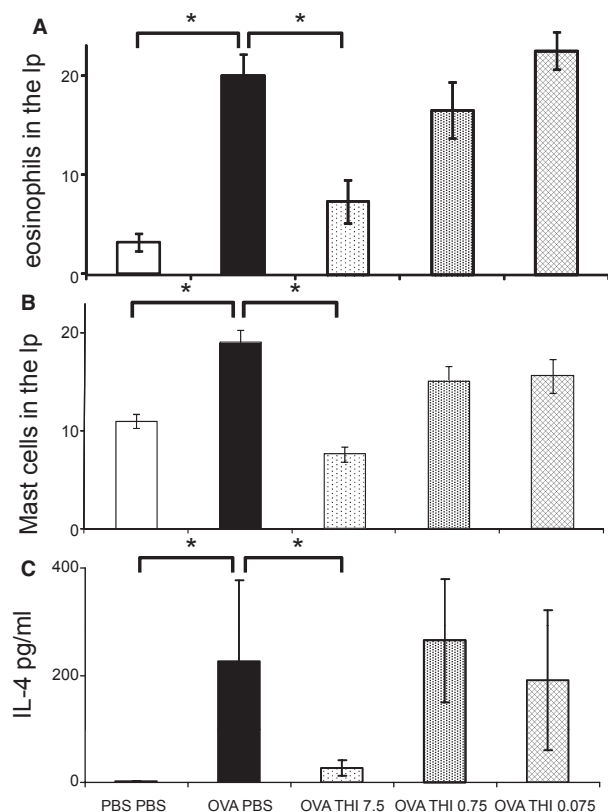


Figure 3 Topical THI treatment reduces allergic airway inflammation in the nasal mucosa of allergic rhinitis mice. (A) The quantification of eosinophil numbers in the lamina propria of the nasal mucosa. (B) Quantification of the number of mast cells in the lamina propria of the nasal mucosa. (C) Cervical lymph nodes (LN) cell suspensions were restimulated *in vitro* for 4 days with ovalbumin (OVA) and assayed for IL-4 production using ELISA assay. Data are shown as mean $\pm$ SEM. * $P < 0.05$; $n = 5$ animals per group.

mucosa tissue expresses Th2 mRNA cytokines IL-5, IL-13, as well as the Treg transcription factor Foxp3 and IL-10, as this pathway seems to be involved and activated by treatment with FTY720 (12). The high level of IL-5 mRNA in the AR group was reduced fivefold after FTY720 treatment (Fig. 1F). Finally, we investigated the effect of FTY720 on enzymes involved in the production of sphingosine and failed to see any alterations in mRNA levels (see Data S1 and Fig. 1).

Induction of adaptive Th2 immunity to inhaled allergens depends on the function of DCs that reside in the mucosal lining. We therefore next evaluated the effects of FTY on dendritic cells. In OVA-alum immunized and OVA-exposed animals AR mice, the strong increase in CD11c⁺ cells in the epithelium and in the lamina propria (Fig. 1G) was strongly reduced by treatment with FTY720 (median 4 cells/mm as opposed to 0.7 cells/mm ($P < 0.02$) in the epithelium and 12.7 as opposed to 4.4 cells/mm ($P < 0.02$) in the lamina propria).

FTY720 treatment reduces eosinophilic inflammation during ongoing inflammation and resolution of allergic inflammation

Next, we attempted to determine whether FTY720 is potent enough to suppress allergic inflammation when AR is already fully established and animals are challenged with an additional series of challenges with OVA mimicking the situation in AR patients. Allergic rhinitis animals and PBS controls were then challenged again on three successive days by instilling a droplet of 10 μ l PBS (PBS PBS control) or OVA (1 mg/ml) (PBS OVA AR) admixed or not with FTY 0.3 mg/ml (FTY OVA AR). To study whether FTY was able to speed up the recovery following the 3-week OVA challenge, some AR mice received PBS plus FTY (FTY PBS AR) or PBS alone (PBS PBS AR) in each nostril with a micropipettor. When FTY treatments were only started 3 weeks after OVA challenges, the FTY-treated animals still had significant reductions in eosinophil numbers: from 22 to 11 in the lamina propria of the nasal mucosa. This reduction was less dramatic in the epithelium (Fig. 2A,B). FTY720 was much more potent in the absence of further OVA challenge, essentially showing it speeded up recovery from eosinophilic inflammation. In the FTY PBS AR groups, eosinophil numbers fell from 18 in the PBS PBS AR group to 7 in the FTY PBS AR.

The T cells obtained from the draining LN had sharply reduced levels of IL-5, IL10 and IL-13 (Fig. 2C–C–G) when animals with already established AR were treated with FTY. Animals sham-challenged with PBS produced low levels of cytokines, suggesting that cytokine production is already completely resolved 1 week after the last of a series of 3-week OVA challenges. No additional treatment effect was therefore observed on T-cell cytokine production with FTY (Fig. 2D–G).

Mast cells accumulate during ongoing AR and are reduced by FTY treatment

In addition to eosinophils, cells staining metachromatically upon toluidin blue staining (i.e. mast cells and basophils Fig. 2H) were evaluated to determine whether they were affected by FTY in mice with already fully established AR. FTY treatment in animals with AR significantly reduced mast cell numbers: from 15.9 to 10.7 in the lamina propria of the nasal mucosa. After 3 weeks of OVA challenges in AR mice, the mast cell numbers turned back to baseline levels in the absence of further OVA challenge. No additional effect of FTY treatment could therefore be observed during this resolution phase of AR.

Inhibition of sphingosine lyase activity by THI abrogates eosinophilic inflammation

To address whether sphingosine-lyase (SL) inhibition is acting to suppress inflammation, some mice were treated with THI at the time of allergen challenge. Dose–response evaluation demonstrated that (Fig. 3A) THI (7.5 μ g) was effective

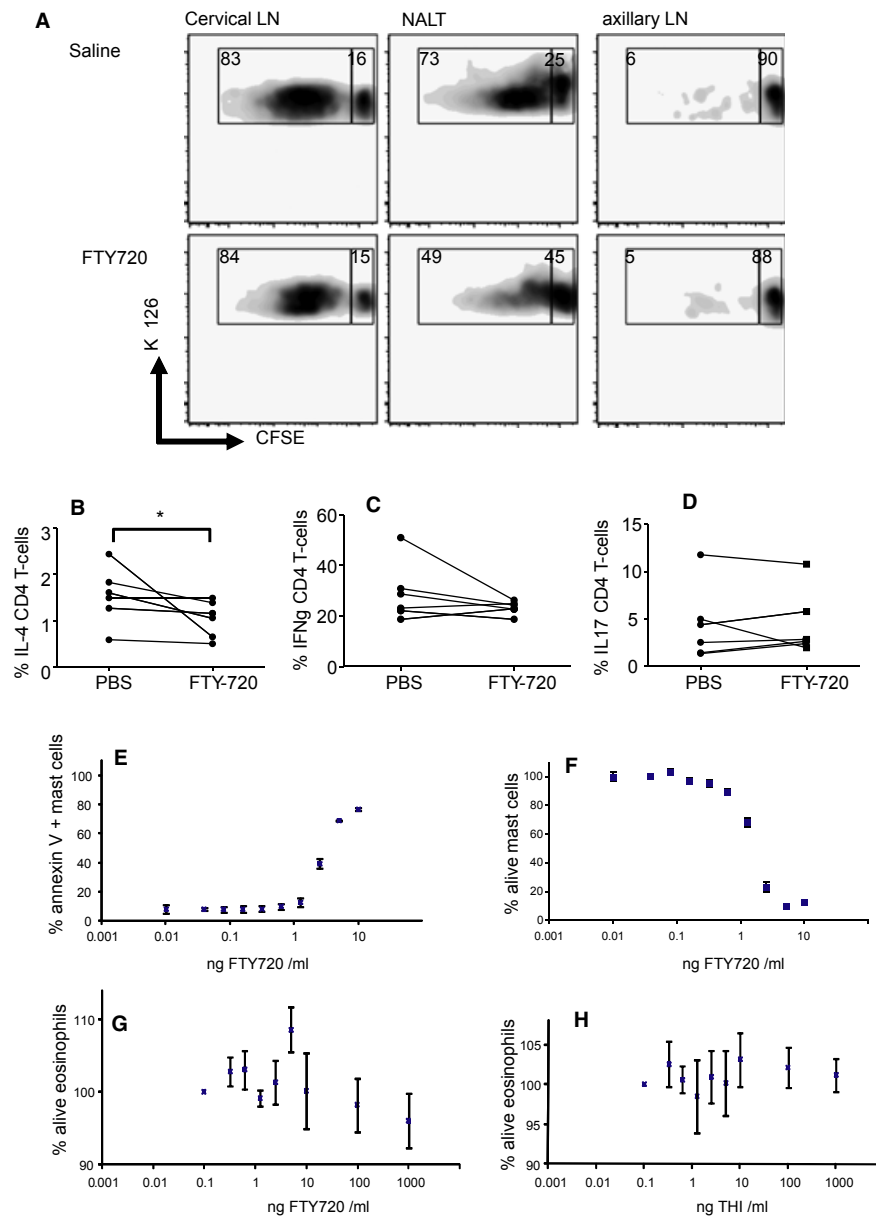


Figure 4 FTY720 inhibits *in vitro* Th2 cell differentiation and *in vivo* T-cell proliferation and served as an inducer of mast cell apoptosis. Naïve animals were given a cohort of CFSE- labeled ovalbumin (OVA)-specific CD4+ TCR transgenic T cells (Kj1.26). This made it possible to trace the occurrence of T-cell proliferation in the lymphatic structures of the shock organ cervical lymph nodes (LN) and nasal-associated lymphoid tissue (NALT). The axillary LNs were used as a control (A). Using this system, which is highly sensitive to the presence of minute amounts of antigen in the absence or presence of FTY720, we only found divisions of CFSE-labeled T cells in the lymphoid structures draining the upper airway, which was blocked in the NALT of FTY720-treated animals when compared to saline-treated animals. Naïve T cells can differentiate into Th2 cells and other T-cell lineages like Th1 and Th17 cells. (B) Th2-cell differentiation is inhibited by FTY720, as shown by the reduction in the proportion of T cells that stains positive for IL-4. No inhibition was observed for (C) the proportion of IFN γ representing Th1 cells or for (D) the proportion of

IL-17 representing Th17 cells. Apoptosis is induced by FTY720 in a concentration-dependent way for bone marrow-derived mast cells. (E) Mast cells are positive for Annexin V, indicating cell apoptosis. (F) The proportion of live mast cells that gradually decrease in relation to higher concentrations of FTY720 ($n = 3$ of one representative experiment of two independent experiments). (G) To address the effects of FTY on eosinophil survival, eosinophil cultures were performed. Balb/c mice bone marrow cells (0.5×10^6 /ml) were grown in the presence of RPMI medium supplemented with 30% fetal calf serum and IL-5 25 ng/ml gentamycin and beta mercaptoethanol. After 6 days of culture cells, FTY or THI was introduced in the appropriate concentration as indicated for 24 h, and cells were analyzed for siglec F (a marker specific for eosinophils) and accumulation of propidium iodide and Annexin V surface staining. The percentage of alive eosinophils treated with FTY (G) and percentage alive eosinophils treated with THI (H) are shown ($n = 5$ animals).

in reducing eosinophilic airway inflammation, while a tenfold lower concentration of 0.75 μg produced some reduction in eosinophilic inflammation. The concentration of 0.075 μg was ineffective and did not reduce airway inflammation. There was no effect of the highest dose of THI treatment on the number of DCs in mice with AR (data not shown). Allergic rhinitis animals treated with 7.5 μg of THI had 7.6 mast cells, as compared to 19 mast cells in the lamina propria of animals treated with saline (Fig. 3B).

Lymphocytes obtained from the cervical LN and restimulated with OVA produced less IL-4 in AR animals treated with THI 7.5 μg than in saline-treated AR animals (Fig. 3C). No THI treatment effect was observed for IL-5, IL-10, IL-13 or IFN γ (data not shown). Thus, THI-mediated inhibition of SL seems to be effective in reducing upper airway inflammation, in a process closely resembling the actions of FTY720.

FTY720 impaired T-cell proliferation and Th2 cell differentiation

The dramatic effects of FTY720 and THI on airway eosinophilia *in vivo* could be explained by direct effects of these compounds on T cells and/or effector cells of the allergic cascade. To address this, we studied the effect of FTY720 on T-cell proliferation *in vivo*. Mice received a cohort of CFSE-labeled DO11.10 OVA-specific CD4⁺ TCR transgenic T cells so that we could trace the occurrence of T-cell proliferation in the draining LN. The axillary LNs were used as a non-draining lymphoid tissue control. We applied the OVA antigen intranasally in the absence or presence of FTY720. Analyzing the cervical LN, the nasal-associated lymphoid tissue (NALT) and the axillary LN showed only divisions of CFSE-labeled T cells in the lymphoid structures draining the upper airway, which was blocked partially in the NALT by FTY720-treatment when compared to saline-treatment (Fig. 4A). This effect was not observed in the cervical nodes.

As the effects of FTY on T-cell proliferation were only partial, we then addressed whether FTY720 affects T-cell differentiation. Cells from whole lymph node and spleen obtained from 6-week-old DO1110 mice were put in culture, under T helper polarizing conditions and in the presence of OVA₃₂₃₋₃₃₉ peptide (see Material and methods) and in the presence of FTY720. Flow cytometric analysis showed a significant reduction in IL-4-producing CD4-positive T-cells cultured under Th2 conditions when FTY720 was added to the culture (Fig. 4B). Strikingly, IFN γ -producing Th1 cells (C) and IL-17-producing Th17 cells (D) were not affected by FTY720 treatment. Therefore, FTY seems to mainly affect Th2 development and partially blocks CD4 T-cell proliferation.

FTY720 induces apoptosis in mast cells, but not eosinophils

In addition to the strong effects on Th2 differentiation, which might explain the strong effects on subsequent airway eosinophilia, we also noticed a strong reduction in MC numbers in AR animals treated with FTY720. As mast cells are not so dependent on T cells, we therefore questioned whether FTY720 might directly affect MC biology. We used an *in vi-*

tro mast cell culture system to study the direct effect of FTY720 in greater detail. MCs were generated by growing bone marrow cells with IL-3 for 5 weeks. MCs characterized as Fc ϵ RI-positive and CD117-positive cells were incubated with FTY720 for 18 h. Levels of apoptotic MCs staining positive for Annexin V (Fig. 4E) increased sharply from 0.6 ng/ml FTY720, and, in line with this, the proportion of dead MCs staining positive for PI also gradually increased from 0.6 ng/ml (Fig. 4F). This observation could explain why numbers of mast cells drop after FTY720 treatment *in vivo*. In contrast, when eosinophils were exposed for 24 h to a concentration up to 1 $\mu\text{g}/\text{ml}$ of FTY720 or THI, no signs of apoptosis (Annexin V staining) were observed in eosinophils (Fig. 4G,H).

Discussion

FTY720 is a prodrug and a member of a new class of immune modulators known as the sphingosine-1-phosphate receptor agonists. Here, we have shown for the first time that FTY720 can reduce upper airway eosinophilic inflammation through the inhibition of Th2 differentiation and by inducing mast cell apoptosis. FTY720 can act as a S1P analogue but can also inhibit the activity of sphingosine lyase. The end result of both processes is an increase in S1P1 receptor triggering. Many of the treatment effects of FTY720 could be mimicked by THI, a drug inhibiting sphingosine lyase directly, suggesting that FTY720 reduces allergic inflammation via both mechanisms. Addition of THI treatment to suboptimally FTY-treated mice could optimize the treatment effect to its maximum, but there were no signs of synergism in this effect (data not shown).

The current data add to a growing list of papers where it was shown that interfering with S1P metabolism or mimicking its effects by S1P1 agonism can suppress allergic airway inflammation (8, 13–16). In some of this work, the proposed mechanism of action of systemically administered S1P interfering drugs is through interference with lymphocyte recirculation. We do not believe that lymphocyte recirculation is the predominant way by which topical FTY720 or THI suppress inflammation. In our previous work employing FTY720 to mice lungs, we have found that it reduced airway DCs synapse formation with effector T cells, and the potential of lung DCs to migrate to the mediastinal LN (8). When we gave FTY720 to the nose of mice, we similarly observed strong effects on the recruitment of DCs to the nose, potentially explaining how FTY suppresses allergy in the nose. Indeed, DCs express sphingosine 1 phosphate receptors 1–5 (S1P1–5) and they play a crucial role in the development and maintenance of AR (3, 8, 17). This suggests that the effect of topical intranasal treatment of FTY may be mediated by at least in part by affecting and impairing the DC function.

The underlying mechanism strongly suggests that T-cell function is altered by FTY720, suppressing Th2 differentiation. Others have suggested that FTY720 supports the induction of Tregs (12, 18, 19). To establish a better insight into how local FTY720 modulates the immune response, we studied its effect on T-cell proliferation and Th differentiation *in*

vitro and *in vivo*. T-cell proliferation was inhibited *in vivo* in the NALT structures but not in the cervical nodes, due to the topical i.n. treatment of FTY720. We also found a clear reduction in the proportion of IL-4-producing T cells in *in vitro* assays of Th differentiation and found a reduction in local Th2 cytokines in the nose-draining LN and nasal tissues of mice treated with FTY720 and/or THI. This observation strongly suggests that FTY720 suppresses the outgrowth of naïve T cells in Th2 cells. In addition to the effects of THI and FTY720 on DCs and T cells, we also found that interfering with S1P1 affected mast cells *in vivo* and *in vitro*. One of the hallmarks of allergic inflammation in humans is the elevated number of mast cells in the nasal mucosa that contribute to both the early and late phase allergic response (20). In this mouse model, we found the same increase in mucosal mast cells in AR. We also found that THI and FTY can both suppress the increase in mast cells in the nasal mucosa of AR mice. Although these effects could have been indirect and caused by a global reduction in airway inflammation, we found evidence for a direct role of FTY720 on mast cells *in vitro*. In bone marrow cultures of mast cells, FTY720 induced their apoptosis. Although this does not formally prove that this would also occur *in vivo*, these are strong indications that FTY720 affects mast cells in addition to its effects on DCs and T cells. However, *in vivo* there were still residual numbers of mast cells after topical FTY720 treatment. As we have not formally discriminated mucosal from connective tissue mast cells, one possibility would be that these two subtypes of mast cells are differentially sensitive to S1P agonism. Alternatively, FTY720 might also act preferentially on newly recruited mast cells, whereas resident mast cells are unaffected. With toluidine blue dye staining, it is impossible to discriminate between 'inflammatory' mast cells and 'resident' mast cells. In conclusion, we found that topical

treatment interfering with S1P metabolism and receptors using THI and FTY720 reduce the features of AR by interfering with DC recruitment, Th2 lymphocyte polarization and survival of mast cells. Studies using topical application of the S1P lyase antagonist THI suggest that these effects might be partly due to inhibition of S1P lyase activity. This study provides preclinical evidence that this topical therapy could be an alternative treatment strategy in treating AR, targeting the very cells that control the disease pathogenesis.

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Author contributions

The study was designed by Alex KleinJan and Bart Lambrecht. Experimental work was performed by Alex KleinJan, Menno van Nimwegen and Karolina Leman. The manuscript was written by Alex KleinJan, Bart Lambrecht and supervised by Henk Hoogsteden.

Conflict of interest

The authors declare that no conflict of interest exists.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and methods.

Figure S1. Quantitative Rt-PCR analyses.

References

- Baraniuk JN. Mechanisms of allergic rhinitis. *Curr Allergy Asthma Rep* 2001;**1**:207–217.
- Peebles RS Jr, Togias A. Role of mast cells and basophils in rhinitis. *Chem Immunol* 1995;**62**:60–83.
- KleinJan A, Willart M, van Rijst LS, Braunstahl GJ, Leman K, Jung S et al. An essential role for dendritic cells in human and experimental allergic rhinitis. *J Allergy Clin Immunol* 2006;**118**:1117–1125.
- Kobayashi N, Terada N, Hamano N, Numata T, Konno A. Transepithelial migration of activated eosinophils induces a decrease of E-cadherin expression in cultured human nasal epithelial cells. *Clin Exp Allergy* 2000;**30**:807–817.
- Yu HQ, Liu ZG, Yu KY, Xu ZQ, Qiu J. Immunotherapy with recombinant house dust mite group 2 allergen vaccine inhibits allergic airway inflammation in mice. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 2006;**24**:414–419.
- van Rijst LS, Jung S, KleinJan A, Vos N, Willart M, Duez C et al. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 2005;**201**:981–991.
- Rosen H, Gonzalez-Cabrera P, Marsolais D, Cahalan S, Don AS, Sanna MG. Modulating tone: the overture of S1P receptor immunotherapeutics. *Immunol Rev* 2008;**223**:221–235.
- Idzko M, Hammad H, van Nimwegen M, Kool M, Muller T, Soullie T et al. Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* 2006;**116**:2935–2944.
- Sawicka E, Zuany-Amorim C, Manlius C, Trifilieff A, Brinkmann V, Kemeny DM et al. Inhibition of Th1- and Th2-mediated airway inflammation by the sphingosine 1-phosphate receptor agonist FTY720. *J Immunol* 2003;**171**:6206–6214.
- Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* 2005;**309**:1735–1739.
- Klein Wolterink RGJ, KleinJan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol* 2012;**42**:1106–1116.
- Daniel C, Sartory N, Zahn N, Geisslinger G, Radeke HH, Stein JM. FTY720 ameliorates Th1-mediated colitis in mice by directly affecting the functional activity of CD4 + CD25 + regulatory T cells. *J Immunol* 2007;**178**:2458–2468.

13. Blazquez AB, Knight AK, Getachew H, Bromberg JS, Lira SA, Mayer L et al. A functional role for CCR6 on proallergic T cells in the gastrointestinal tract. *Gastroenterology* 2010;**138**:275–284 e271–274.
14. Lai WQ, Goh HH, Bao Z, Wong WS, Melendez AJ, Leung BP. The role of sphingosine kinase in a murine model of allergic asthma. *J Immunol* 2008;**180**:4323–4329.
15. Lai WQ, Irwan AW, Goh HH, Howe HS, Yu DT, Valle-Onate R et al. Anti-inflammatory effects of sphingosine kinase modulation in inflammatory arthritis. *J Immunol* 2008;**181**:8010–8017.
16. Sumi T, Fukushima A, Nishino K, Fukuda K, Kumagai N, Nishida T et al. Treatment with FTY720 during the induction or effector phase suppresses the development of experimental allergic conjunctivitis in mice. *Cell Biol Int* 2009;**33**:534–541.
17. Godthelp T, Fokkens WJ, Kleinjan A, Holm AF, Mulder PG, Prens EP et al. Antigen presenting cells in the nasal mucosa of patients with allergic rhinitis during allergen provocation. *Clin Exp Allergy* 1996;**26**:677–688.
18. Goetzl EJ, Liao JJ, Huang MC. Regulation of the roles of sphingosine 1-phosphate and its type 1 G protein-coupled receptor in T cell immunity and autoimmunity. *Biochim Biophys Acta* 2008;**1781**:503–507.
19. Sehrawat S, Rouse BT. Anti-inflammatory effects of FTY720 against viral-induced immunopathology: role of drug-induced conversion of T cells to become Foxp3 + regulators. *J Immunol* 2008;**180**:7636–7647.
20. Fokkens WJ, Godthelp T, Holm AF, Blom H, Mulder PG, Vroom TM et al. Dynamics of mast cells in the nasal mucosa of patients with allergic rhinitis and non-allergic controls: a biopsy study. *Clin Exp Allergy* 1992;**22**:701–710.

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Material and methods

Balb/c female mice aged six to eight weeks were obtained from Harlan (Zeist, Netherlands). OVA-TCR transgenic DO11.10 mice on Balb/c background were used as donors for T cells in adoptive transfer and Th cell cultures. The mice were housed in SPF conditions at the animal care facility of the Erasmus MC Rotterdam with approval from the local animal ethics and welfare committee.

A mouse model of AR

In this study, we used a model for AR as described previously³. In brief, mice (N=4-6 animals per group) were first immunised with ovalbumin (10 µg OVA, grade V from Sigma) emulsified in 2 mg AL(OH)₃ (OVA/alum) in 0.5 ml PBS or as a control with PBS in alum by an intraperitoneal injection on day 0 and day 7. Ten days later, awake mice were challenged by instilling a droplet of 10 µl OVA (1µg/ml) in each nostril with a micropipettor on three successive days a week for three consecutive weeks. Treatment with FTY720 or diluent was given together with the OVA challenge (OVA-FTY and OVA-dil group). The control group was sensitized to OVA but was given challenge with PBS in the presence of diluent (PBS-dil). Twenty-four hours after the last OVA or PBS application, mice were killed using anaesthetic overdose followed by bleeding. The palatine containing the nasal mucosa was snap frozen in freezing solution (TissueTek, Zoeterwoude Netherlands). The cervical lymph nodes were collected. Single cell suspensions were made by pressing lymph nodes through a 100 µm cell sieve.

To study the effect of FTY720 in ongoing inflammation and resolution of allergic inflammation animals were additionally treated after standard induction of allergic rhinitis with three days of OVA challenge as described above in combination with FTY720 treatment or PBS challenge in combination with FTY720 treatment or PBS, to study if FTY720 was able to speed up the recovery following the 3-week OVA challenge.

In vitro lymph node cell restimulation with OVA

Cervical lymph node (LN) single cell suspensions (1x10⁶/ml) were stimulated with 10 µg/ml of OVA in RPMI supplemented with 5% FCS, gentamycin and β-mercaptoethanol for 4 days. Supernatants of the LN cell suspensions were harvested

and stored for ELISA quantification (IL-4, IL-5, IL-10, IL-13 and IFN- γ by commercially available ELISA, BD Biosciences).

Immunohistochemical analysis of the nasal mucosa

Eosinophils and CD11c⁺ dendritic cells were immunohistochemically stained as previously described for single and double staining³. Six- μ m-thick frozen sections (Micronic, Adamas, Netherlands) were stained with rat Abs against mouse MBP (eosinophils, a gift from J. J. Lee, Mayo Clinic, Scottsdale, AZ) or with hamster Abs against CD11c (Clone N418). Frozen sections were warmed to RT, fixed with acetone for 10 minutes and rinsed in PBS. Sections were incubated in peroxidase blocking solution (0.1% H₂O₂ and 0.1% sodium azide in PBS) for 30 minutes and rinsed with PBS. Slides were placed in a semi-automatic stainer (Sequenza) and incubated in 1:10 diluted normal Goat serum (CLB, Amsterdam, Netherlands) for 10 min and subsequently for 60 min with the diluted primary Abs, followed by rinsing in PBS for 5 min and incubation for 30 min with diluted secondary Abs (Goat anti rat conjugated with alkaline phosphatase or goat anti hamster conjugated with peroxidase). After rinsing, slides were incubated with New Fuchsin substrate for alkaline phosphatase conjugates or nova red substrate for peroxidase conjugates. Finally, the sections were rinsed in distilled water, counterstained with Gills triple strength haematoxylin and mounted in vecta mount (Vector). Cells stained positive for MBP when a nucleus in blue and red staining cytoplasm was observed, cells were marked as CD11c positive dendritic cells when cells have a red cytoplasm and a dendritic shape

Mast cells were evaluated with metachromatic, toluidine blue staining. Mast cells stained purple blue with the toluidine blue staining. Frozen sections were histochemically stained with toluidine blue 1% at pH of 0.5 (HCl) for at least 5 minutes and observed immediately.

For quantification of the inflammatory infiltrate, mast cells or eosinophils cells were counted in the entire epithelium and in the submucosa 100 μ m deep in the lamina propria along the basal membrane. To quantify cells, the nasal mucosa was divided into the epithelium and the first 100 μ m depth of the lamina propria. Cell numbers were expressed per millimeters length of basal membrane³. The evaluation of the samples was performed in a blinded fashion.

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96 **In vitro T-helper cell cultures**

97 Single-cell suspensions were prepared by pressing the spleen and lymph nodes from
98 DO11.10 OVA specific TCR Tg mice through a 100 µm cell strainer. Cells were
99 counted and put in culture in 24 wells at 2*10e6 cells/ml, 1 ml per well in complete
100 medium supplemented with OVA₃₂₃₋₃₃₉ peptide in the following conditions: Th1
101 (adding IL-12 10 ng/ml, anti-IL-4 5ug/ml), Th2 (IL-4 10ng/ml, anti-IL-12 5 ug/ml, anti
102 IFN-γ 10ug/ml), Th17 (IL-6 20ng/ml, TGFb 3ng/ml, anti-IL-4 5ug/ml, anti-IFNγ
103 10ug/ml). The effect of FTY720 (1.2µg/ml) on Th cell differentiation was measured by
104 staining for intracellular cytokines (IL-4, IL17 and IFN-γ, representing the T cell
105 lineages Th2, Th17 and Th1 respectively).

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107 **Real-time quantitative reverse transcriptase polymerase chain reaction (Q-Rt-PCR)**

109 Quantitative reverse transcriptase polymerase chain reaction was performed as
110 previously described by our group ¹⁶. Primer sequences for gene , forward primer (F);
111 reverse primer (R), Product length (bp); HPRT, F: GCCCCAAAATGGTTAAGGTT, R:
112 TTGCGCTCATCTTAGGCTTT, 208 bp; Sphingosine kinase 1 (SK1), F:
113 TGTCACCCATGAACCTGCTGTCCCTGCACA, R:
114 AGAAGGCACTGGCTCCTCCAGAGGAACAAG, ? bp; Sphingosine kinase 2 (SK2),
115 F: GGCATTGTCACTGTGTCTGG, R: GCAGAGAAGAAGCGAGCAGT,
116 210 bp; Sphingosine phosphate lyase 1 (S1PLy), F:CTGATGACCGTACCATGTGC,
117 R: GAATCCCTGAGAAGGGGAAG, 169 bp; Sphingosine-1-phosphate phosphatase
118 1 (S1PP1), F: TGGGAGCCATTTCCTAGTTG, R: AGCAAACCTCCCCCAAACCT,
119 156bp; Sphingosine-1-phosphate phosphatase 2 (S1PP2), F:
120 TTTCTCCCTTTCACCCACTG, R: GGCATTCCGTACTCTGCAAT, 176bp; N-
121 acylsphingosine amidohydrolase 2 (neutral ceramidase, nCer), F:
122 CCCTTCATTTGGAACCTTCA, R CTGGATCCGCCAAGATAAAA, 164 bp;
123 Endothelial differentiation sphingolipids G-proteincoupled receptor 1 (Edg1 5 S1P1),
124 F: CTCTGCTCCTGCTTTCCATC, R: GCAGGCAATGAAGACACTCA,
125 173 bp; Endothelial differentiation, sphingolipid G protein–coupled receptor 3 (Edg3 5
126 S1P3), F: GTGTGTTTCATTGCCTGTTGG, R: TTGACTAGACAGCCGCACAC, 208
127 bp; Endothelial differentiation sphingolipids G protein–coupled receptor 5 (Edg5 5

S1P2), F: TCTCAGGGCATGTCACTCTG, R: CAGCTTTTGTCACTGCCGTA, 163 bp;
IL-4, F: CTCATGGAGCTGCAGAGACTCTT, R: CATT
CATGGTGCAGCTTATCGA, 98 bp; IL-5, F: CTCACCGAGCTCTGTTGACAAG, R: CCAATGCATAGCTGGTGATTTTAA, 106 bp;
IL-10, F:GGTTGCCAAGCCTTATCGGA, R:ACCTGCTCCACTGCCTTGCT, 191 bp;
FOXp3, F: CCCAGGAAAGACAGCAACCTT, R: TTCTCACAACCAGGCCACTTG, 89 bp.

Statistical analysis

The distribution of cells in the epithelium and in the lamina propria was not symmetrical and the variances were unequal. For statistical analysis, Kruskal-Wallis one-way ANOVA was used to calculate the overall p-value. A p-value of <0.05 was considered to indicate a significant difference between groups. The nonparametric Mann-Whitney U-test was performed to analyse each group with respect to each other.

Results (supplementary figure 1)

As FTY720 has been shown to have some of its in vivo effects through inhibition of S1P lyase, we wanted to address the effects of local S1P lyase inhibition on suppression of AR. We first evaluated the transcription of the enzymes involved in the production of sphingosine. No significant alterations were observed between PBS PBS, OVA PBS and OVA FTY groups in the mRNA levels for neutral ceramidase, (mnCer), N-acylsphingosine amidohydrolase 2 endothelial differentiation sphingolipids G-protein-coupled receptor 1 (S1P1), endothelial differentiation, sphingolipid G protein-coupled receptor 3 (S1P3) sphingosine kinase 1 (SK1), sphingosine kinase 2 (SK2), sphingosine-1-phosphate phosphatase 1 (S1PP1), sphingosine-1-phosphate phosphatase 2 (S1PP2) and sphingosine phosphate lyase 1 (S1PLy) (see supplementary figure 1).

Supplementair figure 1

QRtPCR of the nasal mucosa

