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Treatment with a sphingosine-1-phosphate analog inhibits airway remodeling following repeated allergen exposure

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Karmouty-Quintana H, Siddiqui S, Hassan M, Tsuchiya K, Risse P, Xicota-Vila L, Marti-Solano M, Martin JG. Treatment with a sphingosine-1-phosphate analog inhibits airway remodeling following repeated allergen exposure. *Am J Physiol Lung Cell Mol Physiol* 302: L736–L745, 2012. First published January 27, 2012; doi:10.1152/ajplung.00050.2011.—Sphingosine-1-phosphate (S1P) is an immunomodulatory lipid mediator that plays an important role in lymphocyte trafficking. Elevated levels of S1P are found in bronchoalveolar lavage (BAL) fluid of patients with asthma; however, its role in disease is not known. FTY720, a synthetic analog of S1P, has been shown to abrogate allergic inflammation and airway hyperresponsiveness following acute allergen challenge. However, its effects on asthmatic airway remodeling induced by repeated allergen exposure are unknown. Ovalbumin (OVA)-sensitized rats were challenged on days 14, 19, and 24 after sensitization. FTY720 or vehicle (PBS) therapy was administered 1 h prior to each challenge. BAL fluid and quantitative histological analysis were performed 48 h after the last challenge. FTY720 inhibited OVA-induced features of airway remodeling including increased airway smooth muscle mass and bronchial neovascularization, without affecting lymphocyte numbers in secondary lymphoid organs. Furthermore, CD3+ cells adjacent to airway smooth muscle bundles were increased in OVA-challenged rats but the increase was inhibited by FTY720. There was an expansion of bronchus-associated lymphoid tissue following FTY720 treatment of OVA-challenged animals. Real-time quantitative PCR revealed that Th2-associated transcription factors were inhibited following FTY720 therapy. Airway remodeling is a cardinal feature of severe asthma. These results demonstrate that allergen-driven airway remodeling can be inhibited by FTY720, offering potential new therapies for the treatment of severe asthma.

bronchus-associated lymphoid tissue; severe asthma; smooth muscle; chronic; T cell

AIRWAY REMODELING IS RECOGNIZED as a cardinal feature of severe asthma. All tissues of the airway wall are involved; most noticeably there is an increase in airway smooth muscle, enhanced epithelial proliferation, apoptosis, goblet cell differentiation, and subepithelial fibrosis (reviewed in Ref. 13). Increased airway smooth muscle has been associated with asthma severity, airway hyperresponsiveness (AHR), fixed airflow limitation, and risk of fatal asthma (3, 7, 17, 28). It has been postulated that an increase in airway smooth muscle mass is sufficient to account for AHR in asthma (19), although loss of the contractile phenotype of remodeled airway smooth muscle could offset the changes in airway responsiveness that would be expected otherwise (18, 24). The mechanisms of airway remodeling are potentially complex and likely also multifactorial. Animal models and observations on human

tissues provide evidence suggesting that T cells in close proximity to airway smooth muscle bundles may increase airway smooth muscle mass in chronic asthma (30, 31, 40, 44, 48).

Patients with severe asthma account for ~10% of all patients with asthma; however, they account for the largest health care costs (14) and highest morbidity affecting children too (6). Current treatment options for patients with severe asthma largely include the use of long-acting β_2 -agonists, corticosteroids, or a combination of these drugs (14). Anti-IgE therapy has become available (16); however, these therapies target the airway tone, the inflammatory cascade, and the immunological response to reexposure to an antigen. They are not able to modulate or reverse the phenomenon of airway remodeling that is now recognized to be an important component of severe asthma (3, 7, 17, 28).

Sphingosine-1-phosphate (S1P) is a bioactive sphingosine metabolite that has been implicated in many cellular responses such as motility, proliferation, survival, angiogenesis, and the trafficking of immune cells (41). The main sources of S1P include platelets (8, 50), erythrocytes (11), mast cells (29), and nonhemopoietic cells such as endothelial cells (2). S1P has been linked to asthma; it is elevated in the BAL fluid of patients with asthma (1). However, its exact role in disease progression is not fully understood. S1P is known to act as a chemoattractant for inflammatory cells particularly lymphocytes (32). However, excessive pharmacological stimulation of the S1P receptor-1 (S1PR1) results in internalization of the S1PR1 and impairment of T cell trafficking (32). Thus S1P may act as a regulator of T cell migration in asthma. This is of particular importance in severe asthma where T cells are potential drivers of the remodeling changes.

FTY720 (fingolimod) is a S1P analog that following phosphorylation *in vivo* (32) is capable of activating all S1P receptors except for S1P2 (26). FTY720 has been shown to possess beneficial effects in experimental autoimmune disorders such as Type 1 diabetes (49) and arthritis (23). However, its greatest potential so far appears to be in the management of multiple sclerosis (45). In models of respiratory disease and asthma, FTY720 has been shown to abrogate airway inflammation following acute allergen (5, 15, 37) or endotoxin challenge (27) in mice. In these models, FTY720 is thought to mediate its effects via sequestration of activated T cells away from sites of inflammation (32). However, whether FTY720 could inhibit the development of structural changes in the lungs associated with chronic asthma is not known. Many of these changes may be mediated by mechanisms that are not necessarily amenable to anti-inflammatory therapies. Our hypothesis is that limiting the capacity of T cells to migrate to the airway smooth muscle bundles would lead to a reduction in airway remodeling and consequently a reduction in AHR to methacho-

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line. Using a synthetic analog of S1P, FTY720, this study aimed to investigate the effects of repeated stimulation of S1PR1 on airway remodeling in a well-characterized model of repeated allergen exposure that presents with hallmarks of chronic asthma (18, 31, 40, 44, 48).

METHODS

Animals. Male Brown Norway (BN) rats (~270 g; Harlan Industries, Indianapolis, IN) were kept at an ambient temperature of $22 \pm 2^\circ\text{C}$ and with a 12-h normal phase light-dark cycle. Drinking water and food pellets were freely available. All experiments were performed with the approval of the Animal Care Committee of McGill University. Sensitization and exposure to ovalbumin (OVA) or vehicle (saline) as well as bronchoalveolar lavage (BAL) and BAL protein concentration were performed as described previously (18, 31, 40, 44, 48). Briefly, rats were sensitized to OVA or PBS on days 0 and 7 and challenged with nebulized OVA (5%) or PBS on days 14, 19, and 21. Experiments were performed on day 23. For FTY720 treatment, rats were lightly anesthetized with 4% isoflurane and FTY720 (1, 10, 30, and 100 $\mu\text{g/kg}$; Cayman Chemicals, Ann Arbor, MI) or saline was

intratracheally instilled 1 h prior to each allergen challenge. The doses of FTY720 were chosen based on published data (15, 37).

Immunohistochemistry and morphometry. Formalin-fixed and paraffin-embedded lung sections 5 μm thick were stained with monoclonal antibodies for α -smooth muscle actin (α -SMA; Sigma-Aldrich, St. Louis, MO) and rabbit polyclonal antibodies to CD3 (Abcam, Cambridge, MA). Goat anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA) were used. The signals of α -SMA and CD3 were developed with Vector Red (Vector Laboratories) and counterstained with Gill's modified hematoxylin (EMD Chemicals, Gibbstown, NJ).

The area of airway smooth muscle and number of bronchial blood vessels were assessed from α -SMA-stained slides. The number of airway wall-immunoreactive CD3 cells was determined independently of the organized collections of lymphoid tissue or bronchus-associated lymphoid tissue (BALT).

Determination of CXCL1, IFN- γ , IL-1 β , IL-4, IL-5, IL-13, and TNF- α levels in BAL fluid. A rat cytokine ultrasensitive immunoassay (Meso-Scale Discovery, Gaithersburg, MD) was used to detect the concentrations of CXCL1, IFN- γ , IL-1 β , IL-4, IL-5, IL-13, and TNF- α in BAL fluid collected from PBS-PBS-, OVA-PBS-, and

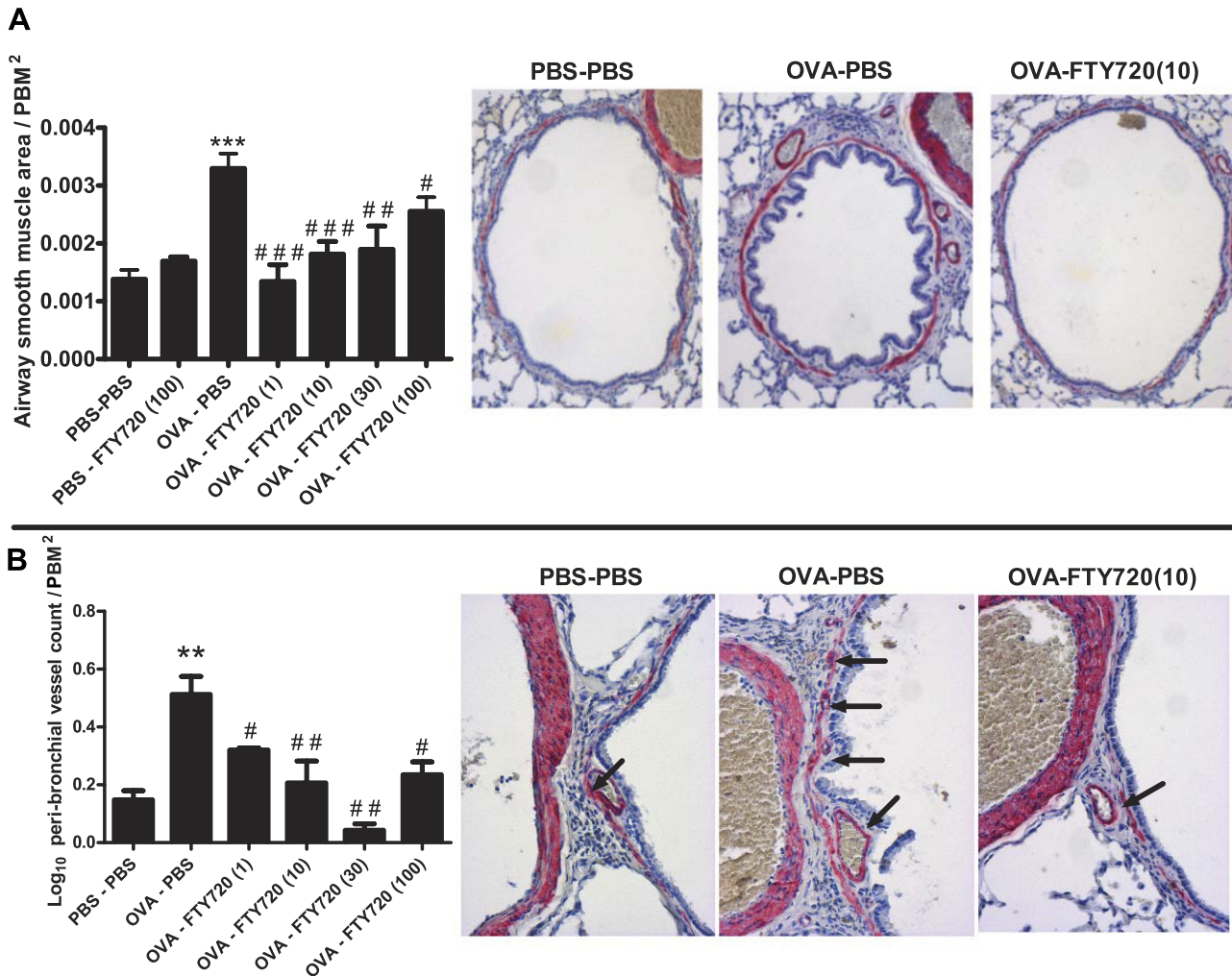


Fig. 1. *A*: airway smooth muscle area assessed from α -smooth muscle actin-immunostained lung sections. *B*: number of bronchial vessels counted from α -smooth muscle actin immunostained lung sections. Sensitized rats challenged with ovalbumin (OVA) (or PBS) on days 14, 19, and 21 and pretreated with FTY720 (1, 10, 30, or 100 $\mu\text{g/kg}$ it) or its vehicle (PBS) 1 h prior to each challenge. Lung sections were stained 48 h after the last challenge. Representative histological images of an airway from a PBS-PBS, OVA-PBS, and OVA-FTY720 10 $\mu\text{g/kg}$ -treated rat are shown. The significance levels * $P < 0.05$ and *** $P < 0.001$ refer to differences with respect to the PBS-PBS group. The significance levels # $P < 0.05$ and ### $P < 0.001$ refer to differences with respect to the OVA-PBS-treated animals. The 1-way ANOVA with the Newman-Keuls multiple-comparison test was performed for all comparisons. PBM², perimeter of the basement membrane, squared.

OVA-FTY720-treated groups. The plate was read on the MSD detector (Sector Imager 2400, MSD, Gaithersburg, MD). Standard curves were drawn by using the manufacturer's software and cytokine concentrations were determined by interpolation.

Lymph node cell count and CD4⁺ T cell migration assay. The cervical (non-lung-draining) secondary lymph nodes were excised from euthanized rats, and the total lymph node cell numbers and the number of isolated CD4⁺ T cells were performed by immunomagnetic selection (31). Cell migration assays were obtained by using a modified Boyden chamber (Neuroprobe, Gaithersburg, MD).

Quantitative RT-PCR. Quantitative RT-PCR was performed from lung homogenates in 96-well plates with the ABI StepOne PCR machine (Applied Biosystem). Gene expression was determined for the following genes: interleukin (IL)-5, IL-13, GATA3, Tbet, S1PR1, and vascular endothelial growth factor (VEGF) by use of SYBR Green PCR Master Mix. Analysis and quantification were

performed by the comparative $\Delta\Delta C_t$ method. S9 was used as housekeeping gene.

Measurement of airway responsiveness to inhaled methacholine. Airway responses to methacholine (Sigma Chemical, St. Louis, MO) were assessed with the flexiVent system (Scireq, Montreal, QC, Canada) at 48 h after the last of three OVA or PBS challenges. Animals were anesthetized with xylazine (10 mg/kg) and pentobarbital (35 mg/kg), and paralysis was induced with 1 mg/kg pancuronium bromide intraperitoneally (Sandoz Canada). The animals were placed on a heating pad, and their temperature was monitored to ensure that it remained between 34 and 36°C. Positive end-expiratory pressure was maintained between 2 and 2.5 cmH₂O, and the animals were ventilated at a tidal volume of 8 ml/kg and a breathing frequency of 90 breaths/min.

Statistical analysis. For the in vivo studies, a one-way analysis of variance (ANOVA) with a Newman-Keuls post hoc test was per-

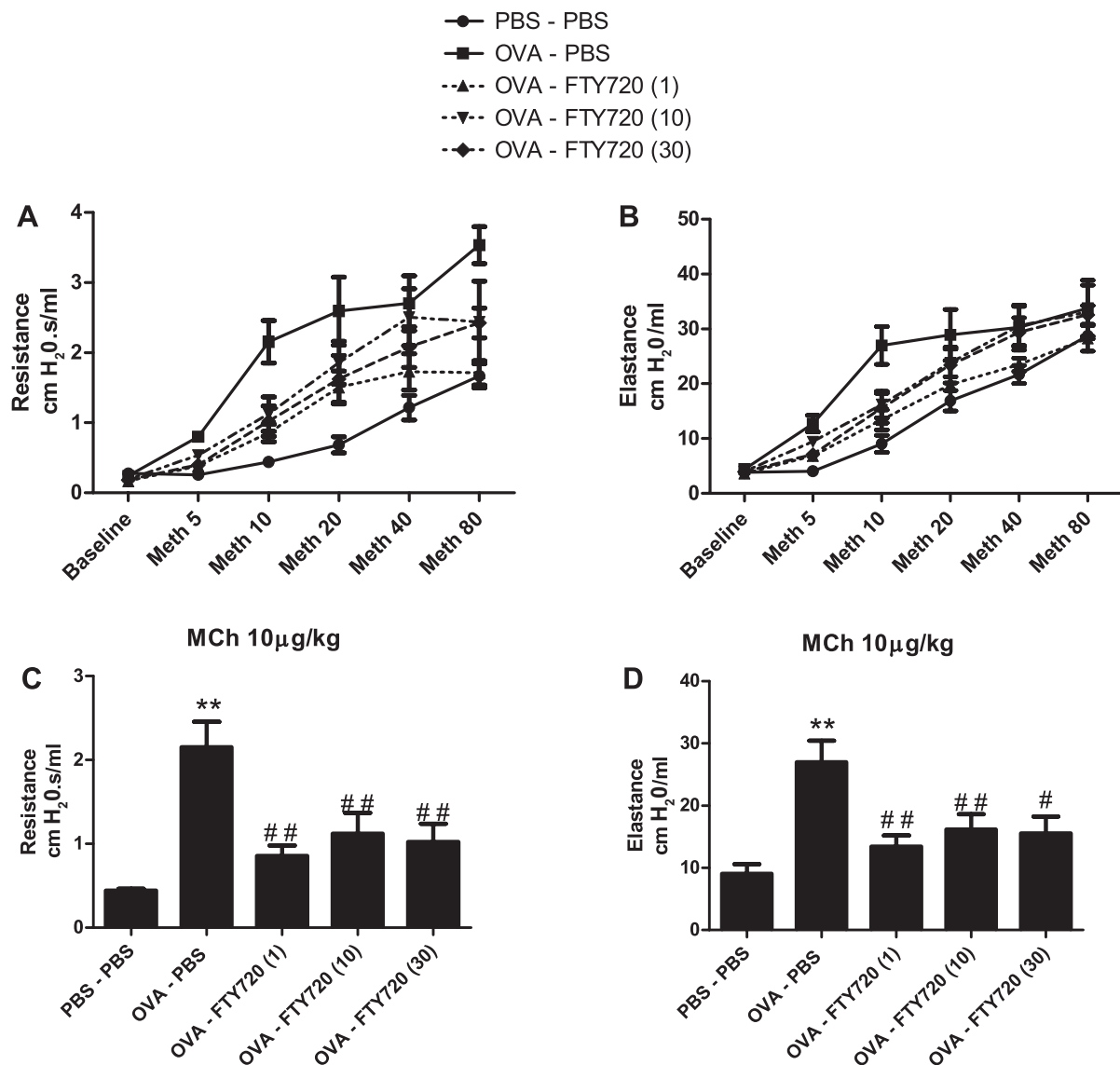


Fig. 2. Lung function measurements following a dose response to nebulized methacholine (MCh) at doses of 5, 10, 20, 40, and 80 µg (Meth 5 to Meth 80) showing resistance (A) and elastance (B) and from sensitized rats challenged with OVA (or PBS) on days 14, 19, and 21 and pretreated with FTY720 (1, 10, or 30 µg/kg it) or its vehicle (PBS) 1 h prior to each challenge. C and D show respiratory system resistance and elastance for all groups for the 10 µg/kg methacholine dose. The significance levels **0.001 < P < 0.01 refer to differences with respect to the PBS-PBS group. The significance levels #P < 0.05 and ##0.001 < P < 0.01 refer to differences with respect to the OVA-PBS-treated animals. The 1-way ANOVA with the Newman-Keuls multiple-comparison test was performed for all analyses.

formed. Statistical significance was defined as $P \leq 0.05$ by use of GraphPad Prism version 5 (GraphPad Software, La Jolla, CA).

RESULTS

Effects of FTY720 on airway smooth muscle remodeling and bronchial angiogenesis. We first examined the potential of S1PR stimulation with FTY720 to prevent the increase in airway smooth muscle observed following repeated exposure to OVA. In this model, as previously described (18, 31, 40, 44, 48), repeated OVA but not PBS challenges led to an increase in airway smooth muscle mass observed morphometrically on α -SMA-stained sections. This effect was inhibited following treatment with FTY720 (1, 10, 30, or 100 $\mu\text{g/kg}$) 1 h prior to each OVA challenge (Fig. 1A), with the dose of 10 $\mu\text{g/kg}$ eliciting the greatest effect. Furthermore, we observed an increase in bronchial angiogenesis following repeated allergen exposure that was inhibited also by pretreatment with FTY720 (Fig. 1B).

Effect of FTY720 on airway hyperresponsiveness. Rats subjected to multiple challenges of OVA had increased AHR to methacholine compared with PBS-challenged rats (Fig. 2). Airway resistance and elastance were significantly affected in OVA-challenged rats (Fig. 2, A–C). Pretreatment with FTY720 at doses of 1, 10, and 30 $\mu\text{g/kg}$ it inhibited the changes in airway function induced by repeated allergen challenge (Fig. 2, A and B). The effects of FTY720 on airway resistance and elastance following 10 $\mu\text{g/kg}$ methacholine challenges clearly show a significant inhibition of increased resistance and elastance induced by OVA (Fig. 2, C and D). The effect on FTY720 on AHR appeared to correlate with the extent of airway smooth muscle mass observed histologically, with a R^2 of 0.937 ($P < 0.0079$; Fig. 3).

Effects of FTY720 on T cell distribution in the airways. T lymphocytes in the vicinity of airway smooth muscle bundles have been postulated to contribute to the development of increased airway smooth muscle mass (20, 30, 31). In this study, using CD3-stained sections, we report an increase in T cells adjacent to or within the airway smooth muscle layers in

rats that were repeatedly challenged with allergen but not PBS (Fig. 4A). Pretreatment with FTY720 (1, 10, 30, or 100 $\mu\text{g/kg}$) 1 h prior to each allergen challenge significantly diminished the number of T cells adjacent to the airway smooth muscle bundles (Fig. 4A). Furthermore, we report that, following repeated allergen challenge, prominent T cell aggregates and BALT were apparent in CD3-stained sections (Fig. 4B), consistent with the development of these structures in instances of chronic inflammation (4, 12, 25). The magnitude of these structures increased dose dependently following FTY720 treatment (Fig. 4B).

Effects of FTY720 treatment on lymph node cell count and CD4+ T cell migration. FTY720 is known for its capacity to cause lymphocytopenia (33), following systemic administration that is attributable to sequestration of lymphocytes in lymph nodes (22, 32). To study the effects of intratracheal administration of FTY720 on lymphocyte migration we performed analysis of non-lung-draining secondary lymph nodes. The observations revealed no significant changes in the number of total lymph node cells and isolated CD4+ cell counts between the OVA-PBS and OVA-FTY720 (100 $\mu\text{g/kg}$) treatment groups (Fig. 5, A and B). Subsequent migration assay experiments revealed that cells from rats treated with FTY720 (100 $\mu\text{g/kg}$) displayed a reduced chemotactic index to S1P compared with lymphocytes harvested from PBS-treated animals (Fig. 5C).

Cytokine expression levels. We next aimed to determine the extent of T cell differentiation following repeated allergen exposure and FTY720 therapy. RT-PCR analysis of lung homogenates demonstrated that multiple OVA challenges resulted in a significant increase in IL-5, IL-13, GATA-3, and S1PR1 (Fig. 6, A–C), but no effects on T-bet and VEGF levels. Treatment with FTY720 (1, 10, or 30 $\mu\text{g/kg}$) resulted in a reduced expression of IL-5, IL-13, GATA3 and S1PR1 (Fig. 6, A–D).

BAL fluid cell and proinflammatory cytokine analyses. FTY720 has been reported to inhibit inflammatory cell influx in acute models of inflammation such as allergen or endotoxin challenge (15, 27, 37). In this study we report an increase in inflammatory cell influx (total cell counts, lymphocytes, macrophages, eosinophils, and neutrophils) following repeated OVA but not PBS challenges (Fig. 7, A–D). Pretreatment with FTY720 (1, 10, 30, or 100 $\mu\text{g/kg}$), 1 h prior to each OVA challenge, inhibited the infiltration of lymphocytes and eosinophils (Fig. 7, B and C). Macrophage infiltration to the airways was only inhibited by FTY720 at 100 $\mu\text{g/kg}$.

High-sensitivity immunoassays from BAL fluid confirmed that repeated OVA challenges led to elevated levels of IL-5 that were inhibited by FTY720 (Fig. 8A). Additionally, an increase in CXCL1 and protein concentration in BAL fluid was apparent following allergen but not PBS challenge (Fig. 8, B and C). Treatment with FTY720 attenuated the increase of CXCL1 (Fig. 8A) and total BAL fluid protein concentrations (Fig. 8, B and C). No significant changes were observed in IFN- γ , IL-1 β , IL-4, IL-13, and TNF- α levels among treatment groups (data not shown).

DISCUSSION

In the present study we have used repeated OVA challenges of actively sensitized rats to evoke asthmatic airway remodeling characterized by increased airway smooth muscle mass and

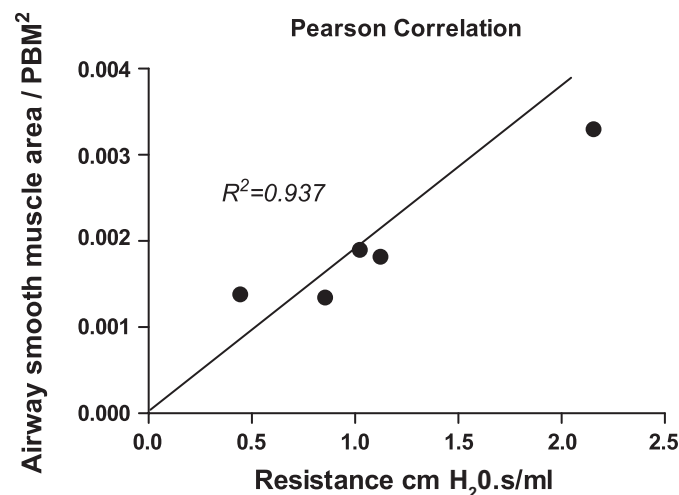


Fig. 3. Pearson correlation plot with a coefficient of correlation (R^2) of 0.937 for airway smooth muscle area/PBM² vs. respiratory resistance (cmH₂O.s·ml⁻¹) values following 10 $\mu\text{g/kg}$ of methacholine challenge for control, OVA-PBS-, and OVA-challenged mice treated with FTY720 (1, 10, and 30 $\mu\text{g/kg}$).

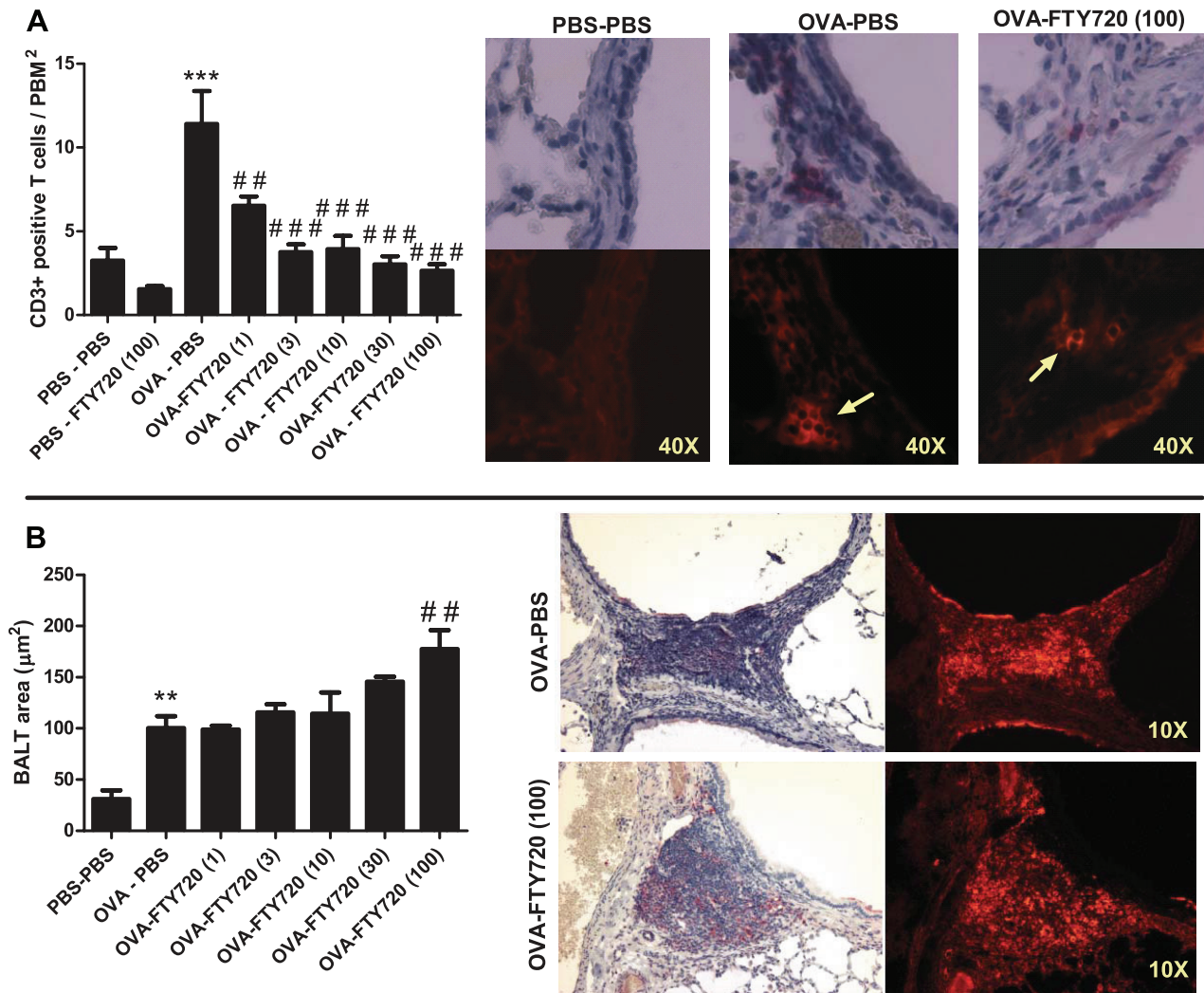


Fig. 4. *A*: number of CD3-positive cells counted adjacent to or within smooth muscle bundles [not including lymphoid clusters or bronchus-associated lymphoid tissue (BALT)]. *B*: area of the BALT. Results were quantified from CD3-immunostained sections from sensitized rats challenged with OVA (or PBS) on days 14, 19, and 21 and pretreated with FTY720 (1, 10, 30, or 100 μg/kg it) or its vehicle (PBS) 1 h prior to each challenge. Lung sections were stained 48 h after the last challenge. Representative histological images showing both bright-field and fluorescent images of the airway lumen and BALT areas from PBS-PBS (except for BALT where none was present), OVA-PBS, and OVA-FTY720 (100 μg/kg)-treated rats are shown; the arrows indicate CD3+ cells. The significance levels **0.001 < *P* < 0.01 and ****P* < 0.001 refer to differences with respect to the PBS-PBS group. The significance levels ##0.001 < *P* < 0.01 and ###*P* < 0.001 refer to differences with respect to the OVA-PBS-treated animals. The 1-way ANOVA with the Newman-Keuls multiple-comparison test was performed for all comparisons.

AHR. These observations are consistent with previous studies in which airway remodeling was elicited following multiple challenges with allergen, in actively sensitized BN rats (18, 31, 40, 44, 48). In addition, we report that multiple allergen challenges lead to bronchial neovascularization, consistent with similar observations in the rat (46) and the mouse (9, 21). The mechanisms behind bronchial neovascularization appear to be independent of VEGF, since transcript levels of this mediator were not changed following repeated OVA challenges. We also demonstrate that multiple allergen challenges lead to the formation of the BALT, recognized as a tertiary lymphoid organ (10), that is present following chronic inflammation in humans (4) and rodents (12, 25). In our study, intratracheal treatment with FTY720 1 h prior to allergen challenge was able to inhibit most features of airway remodeling including increased airway smooth muscle mass and bronchial neovascularization as well as AHR and airway in-

flammation. Interestingly, our data show that increased smooth muscle mass correlates with increased airway hyperresponsiveness, providing further support for increased smooth muscle remodeling leading to worsening of lung function (13). As previously published (30), we believe that the mechanisms leading to excess smooth muscle deposition are large as a result of proliferation of smooth muscle (30, 31). Although increased airway smooth muscle mass, airway inflammation, and epithelial remodeling following multiple OVA challenges are phenomena that have been well characterized (31, 35, 36), their mechanisms are not fully understood. There is growing evidence for the participation of lymphocytes in the induction of the remodeling processes (31). In the present study, we report an increase in airway smooth muscle mass, AHR, and bronchial neovascularization that are associated with an increased presence of lymphocytes, observed in CD3-stained sections, adjacent to airway smooth muscle bundles. Following intratra-

cheal treatment with FTY720, we report a reduction of airway smooth muscle mass, AHR, and neovascularization. These changes were accompanied by a reduction in lymphocytes adjacent to the airway smooth muscle bundles, observed histologically, in BAL fluid and a reduction in markers of Th2 cell

differentiation observed by RT-PCR. These observations are consistent with a postulated role for activated T cells in modulating structural changes in the lungs of patients with asthma. No definitive link of Th2 cells to airway remodeling have been made although recently Th2 cells have been shown to express the epidermal growth factor ligand amphiregulin (51).

Additionally, CD3+ staining revealed the formation of BALT in the lung of OVA- but not PBS-challenged rats. This is consistent with human data showing that the BALT is largely absent in healthy adults (47) but becomes apparent following infection and chronic inflammation both in humans and in murine models (4, 12, 25). The BALT is recognized as a tertiary lymphoid organ (10). Interestingly, in our study, treatment with FTY720 led to an increase in the number and size of the BALT in OVA-challenged rats, without significantly altering the number of cells present in the secondary lymph nodes. This is of particular interest, since several studies have reported a decrease in the number of lymphocytes present in lung-draining lymph nodes following systemic FTY720 treatment correlating with an increase in non-lung-draining lymph nodes (15, 37). These effects are mediated by the phosphorylated form of FTY720 through internalization of the S1P1 receptor on the cell surface (22), rendering the cell unable to respond to chemotactic gradients. Reduced responses to FTY720 in the migration assays of CD4+ cells harvested from FTY720-treated animals is consistent with interference with *in vivo* migration of T cells by FTY720 and with previously published results (38). Our findings suggest that FTY720 following intratracheal administration leads to sequestration of T cells in the BALT and not in secondary lymph nodes, preventing their egress from this tertiary lymphoid organ to the adjacent lung tissue. This could be of important clinical significance for therapies aimed at preventing T cell egress from the BALT or other tertiary lymphoid organs as well future inhalation drug delivery applications of FTY720 or other sphingosine receptor analogs.

RT-PCR analysis of lung homogenates demonstrated that following OVA challenge, the Th2-associated transcription factor GATA-3 and the GATA-3 dependent genes IL-5 and IL-13 were upregulated consistent with the Th2 bias in allergic disease. In support of this, increased levels of IL-5 and eosinophils were observed in the BAL fluid of BN rats exposed to allergen. IL-5 is well known for its effects in prolonging survival of the eosinophil and increasing its effector function (42, 43). Allergen challenge resulted in the increase in the expression of the S1PR1, a phenomenon consistent with the migration of T cells (32) and correlating with the appearance of the BALT. Taken together, these observations suggest the BALT as a site for antigen presentation following allergen challenge of the airways, consistent with evidence suggesting that tertiary lymphoid organs are sites of antigen presentation

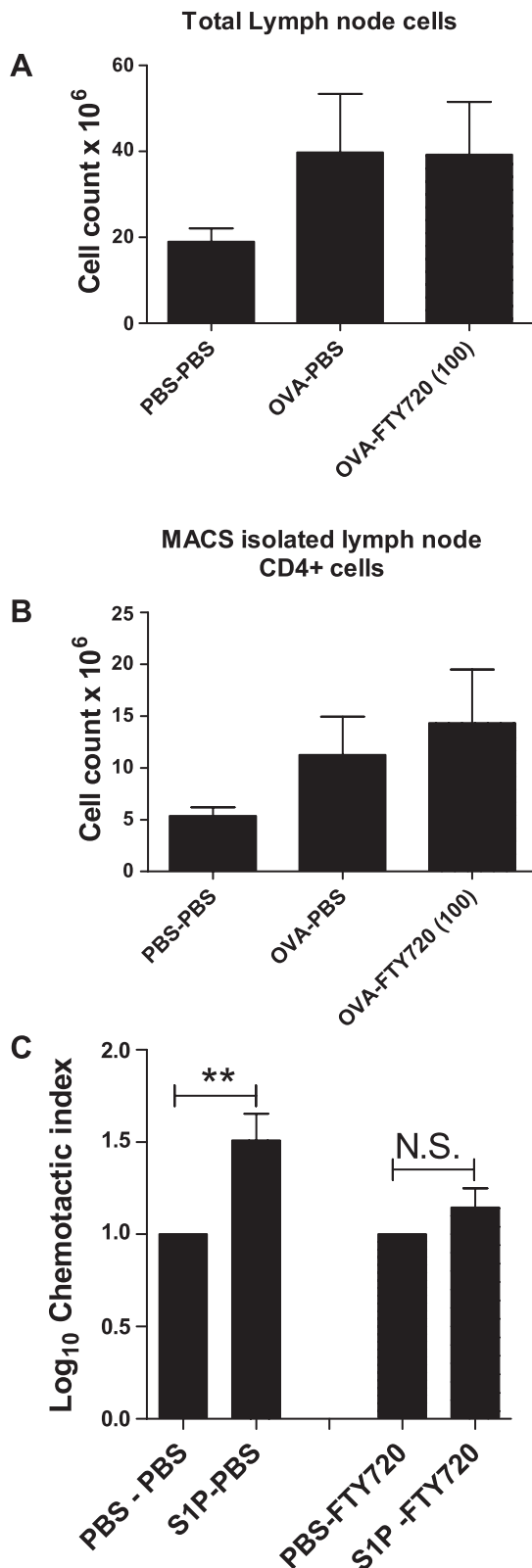


Fig. 5. Cells were isolated by negative immune-magnetic cell separation from the cervical lymph nodes of OVA-sensitized and -challenged rats (5% OVA nebulized for 5 min on days 14, 19, and 24 postsensitization) and pretreated with FTY720 (100 μ g/kg it) or its vehicle (PBS). Total lymph node cell count (A) and total number of CD4+ isolated cells from the lymph node (B) from 3–4 rats challenged with PBS or OVA and treated with FTY720 (100 μ g/kg it) or its vehicle. C: chemotactic index for CD4+ cells toward sphingosine-1-phosphate (S1P, 100 nM). N.S., not significant. MACS, magnetic activated cell sorting.

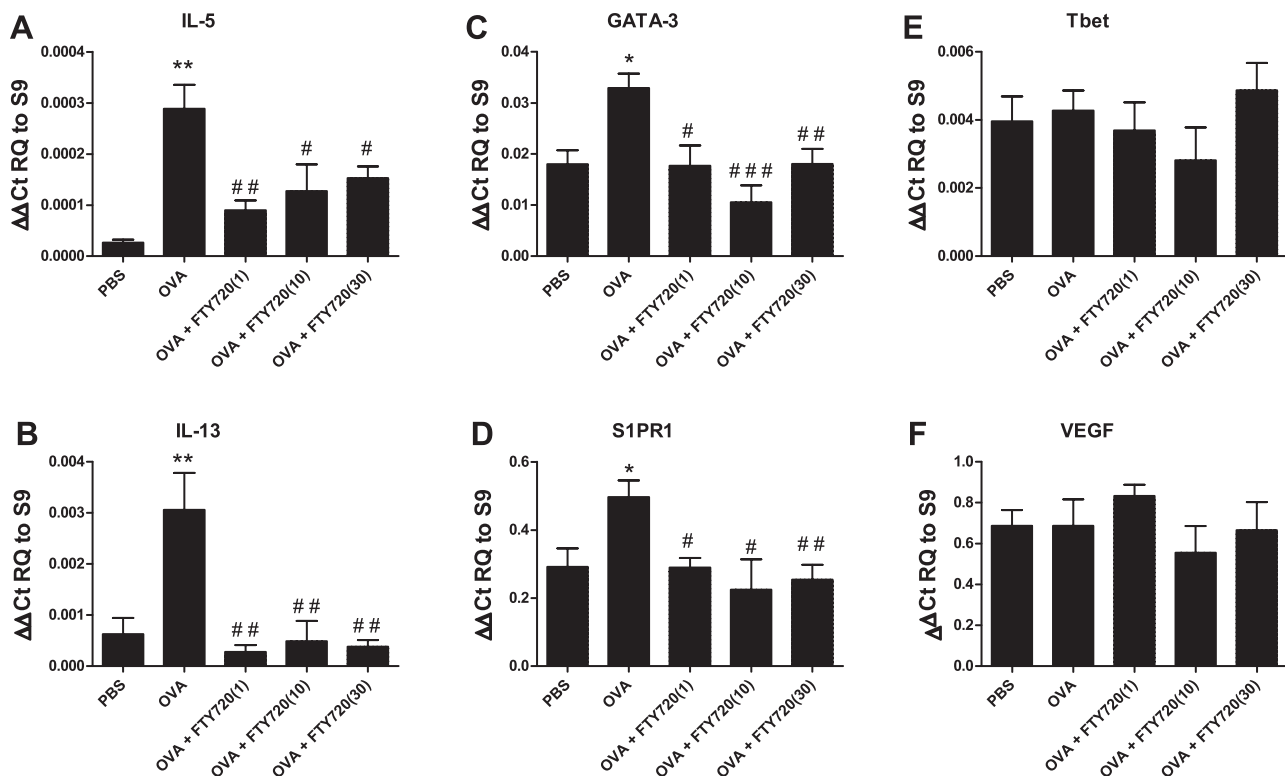


Fig. 6. Expression levels of mRNA for IL-5 (A), IL-13 (B), GATA-3 (C), S1P receptor subtype 1 (S1PR1; D), Tbet (E), and VEGF (F) from sensitized rats challenged with OVA (or PBS) on days 14, 19, and 21 and pretreated with FTY720 (1, 10, or 30 $\mu\text{g/kg}$ it) or its vehicle (PBS) 1 h prior to each challenge. The significance levels * $P < 0.05$ and ** $0.001 < P < 0.01$ refer to differences with respect to the PBS-PBS group. The significance levels # $P < 0.05$, ## $0.001 < P < 0.01$, and ### $P < 0.001$ refer to differences with respect to the OVA-PBS-treated animals. The 1-way ANOVA with the Newman-Keuls multiple-comparison test was performed for all analyses. RQ, relative quantification.

(25). Treatment with FTY720 resulted in the reduction in the expression levels of GATA-3, IL-5, IL-13, and S1PR1. A reduction in IL-5 levels in BAL was apparent following FTY720 treatment, correlating with a reduced eosinophil cell infiltration to the airways and a reduction in total protein content in the BAL.

Several studies have demonstrated that FTY720 is able to prevent inflammation following acute allergen challenge in the mouse (5, 15, 37). The mechanism of action appears to be mediated through the engagement of the S1P1 receptor (5). In the present study we show that inflammation evoked following repeated allergen challenge is inhibited by FTY720. However, contrary to our studies, the models studied involve acute allergen exposure where structural changes in the lung involving increased smooth muscle mass have not been reported.

It is important to consider that S1P mediates a variety of effects on airway smooth muscle. S1P has been shown to induce contraction of airway smooth muscle cells (34) and to stimulate proliferation of these cells directly (1). These observations may help explain why FTY720 inhibits the OVA-induced increase of airway smooth muscle mass best at low doses of 1 and 10 $\mu\text{g/kg}$. This phenomenon is also observed in lung function data, where the inhibition of AHR to methacholine appears to have the greatest effect at doses of 1 and 10 $\mu\text{g/kg}$ FTY720. A recent study by Shea et al. (39) demonstrated that prolonged exposure of S1PR1 agonists including FTY720 at doses between 500 and 5,000 $\mu\text{g/kg}$ resulted in increased fibrosis, vascular leak, and mortality following acute

lung injury. These observations suggest that prolonged exposure to S1PR1 receptor agonists in high concentrations may lead to unwanted effects. These observations in combination with those published by Shea et al. warrant further research into the effects of repeated exposure of S1P1 receptor agonists in the lung.

Airway remodeling is an important phenomenon that leads to cellular, molecular, and structural changes in the lung that compromise its function. S1P may play an important role in modulating the remodeling process in the lungs by regulating T cell trafficking via the S1PR1. However, as shown by Shea et al. (39), prolonged engagement of S1PR1 may result in detrimental effects in the lung. Thus understanding the mechanisms that control the balance of S1P and its receptors in the lung is important for disease pathogenesis and resolution. In conclusion, these results demonstrate that structural changes induced by repeated allergen exposure are mediated by the ensemble of inflammatory cells and their mediators eliciting changes to structural components of the airway. FTY720 therapy is capable of inhibiting the structural changes evoked by repeated allergen exposure. These results suggest that targeting the S1PR1 may be beneficial to patients with severe asthma where reversal and further prevention of airway remodeling may lead to clinical gains. However, it is important to be mindful that several S1P receptors are expressed in many cells of the lungs, such as airway smooth muscle cells and that activation of these receptors may promote unwanted effects including directly mediated remodeling changes.

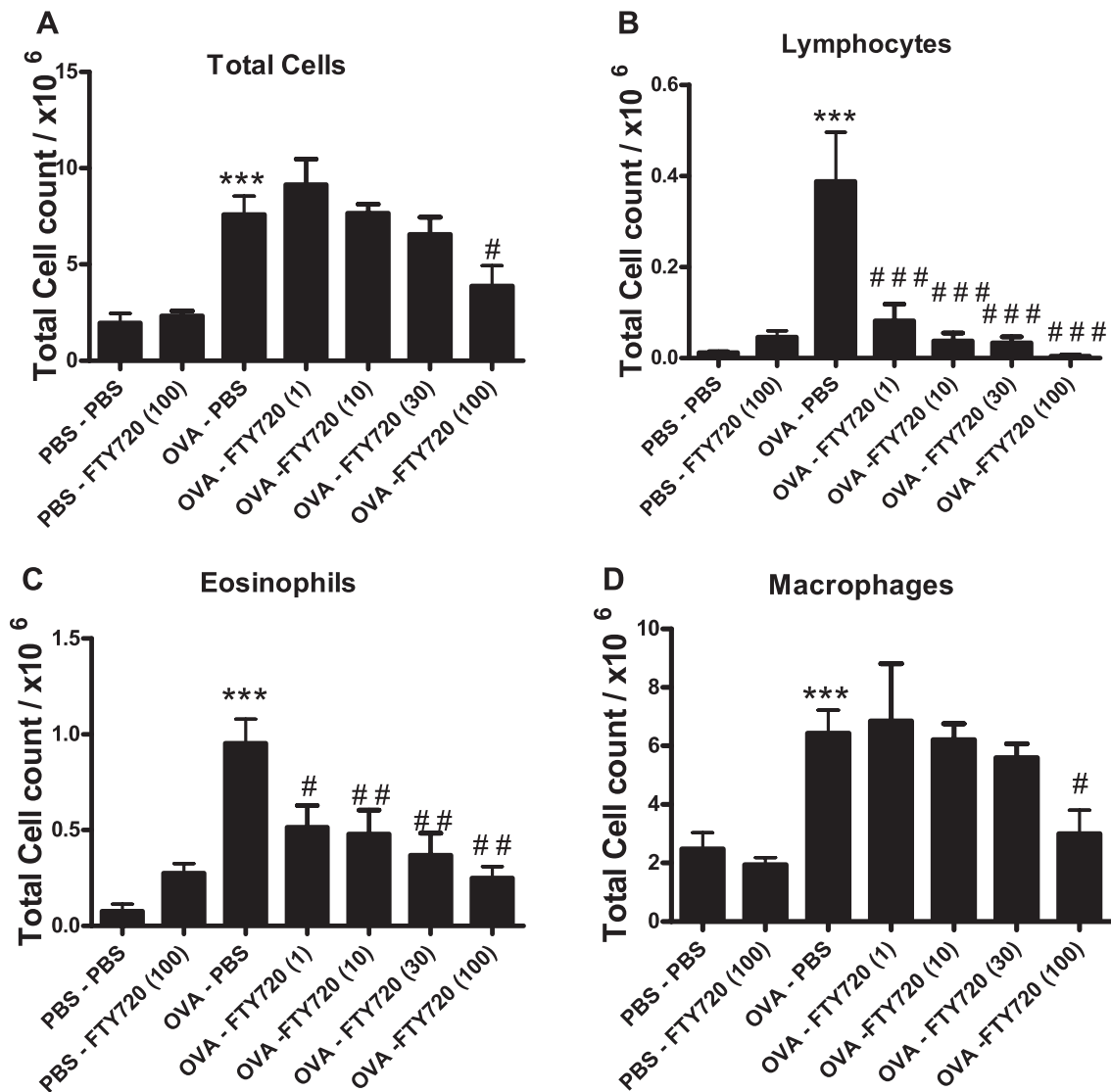


Fig. 7. Bronchoalveolar lavage (BAL) fluid analyses of sensitized rats challenged with ovalbumin (5% solution administered intratracheally for 5 min) or its vehicle (PBS) on days 14, 19, and 24 after sensitization. Rats were pretreated with FTY720 (1, 10, 30, or 100 $\mu\text{g/kg}$ it) or its vehicle (PBS) 1 h prior to each OVA or vehicle challenge ($n = 4-6$ for each treatment). BAL fluid analysis was performed 48 h after the last challenge. The panels show total inflammatory counts (A), lymphocytes (B), eosinophils (C), and macrophages (D). The significance level *** $P < 0.001$ refers to differences with respect to the PBS-PBS group. The significance levels # $P < 0.05$, ## $0.001 < P < 0.01$, and ### $P < 0.001$ refer to differences with respect to the OVA-PBS-treated animals. The 1-way ANOVA with the Newman-Keuls multiple-comparison test was performed for all comparisons.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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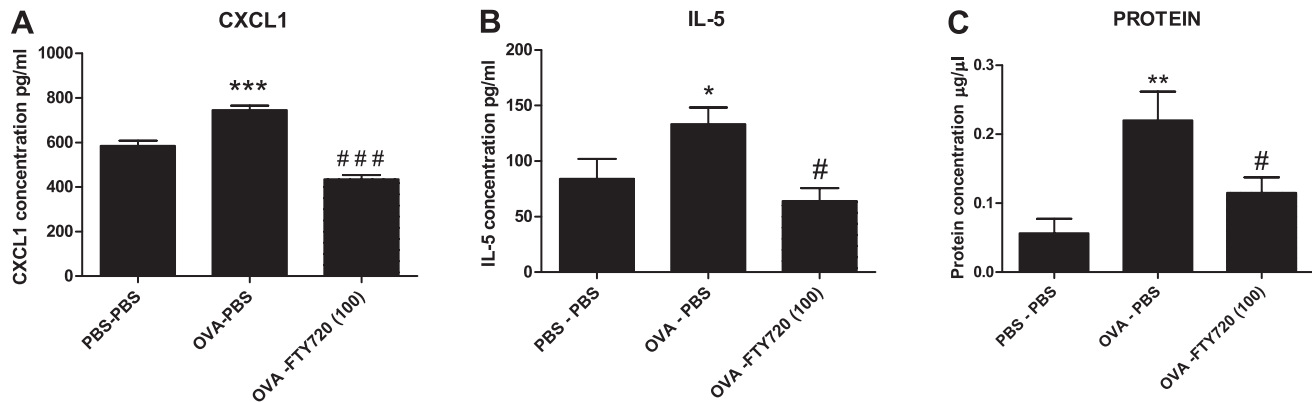


Fig. 8. BAL fluid analyses for CXCL1 (A), IL-5 (B), and total protein concentration (C) from sensitized rats challenged with ovalbumin (5% solution administered intratracheally for 5 min) or its vehicle (PBS) on days 14, 19, and 24 after sensitization. Rats were pretreated with FTY720 (10 or 100 μg/kg it) or its vehicle (PBS) 1 h prior to each OVA challenge. BAL fluid analysis was performed 48 h after the last challenge. The significance levels * $P < 0.05$, ** $0.001 < P < 0.01$, and *** $P < 0.001$ refer to differences with respect to the PBS-PBS group. The significance levels # $P < 0.05$ and ### $P < 0.001$ refer to differences with respect to the OVA-PBS-treated animals. The 1-way ANOVA with the Newman-Keuls multiple-comparison test was performed for all comparisons.

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