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RESEARCH PAPER

Activation of the lung $S1P_1$ receptor reduces allergen-induced plasma leakage in mice

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Background and purpose: It has been suggested that intratracheal administration of the immunomodulator, FTY720, could have anti-inflammatory effects without causing a decrease in blood lymphocyte counts. However, the receptor responsible for this effect has not been defined.
Experimental approach: We have described, in a mouse model of allergen-induced inflammation, the use of proton magnetic resonance imaging to non-invasively assess lung fluid accumulation and inflammation. Here, we used this model to investigate the sphingosine-1-phosphate (S1P) receptor responsible for the anti-inflammatory effect of FTY720.
Key results: When given intranasally, FTY720 (3 and 10 $\mu g \cdot kg^{-1}$) inhibited by approximately 50% the allergen-induced accumulation of fluid in the lung detected by magnetic resonance imaging, but had no effect on the cellular inflammation in the airway space or on circulating blood lymphocytes. Inhibition of the infiltration of inflammatory cells into the airways was only observed at a dose of FTY720 that induced lymphopenia (100 $\mu g \cdot kg^{-1}$). Similar results were observed in $S1P_3$-deficient mice. The effect of FTY720 was mimicked by intranasal treatment of wild-type mice with a $S1P_1$-specific agonist, AUY954.
Conclusions and implications: Thus, in contrast to previously published work, our results suggest that systemic exposure of FTY720 is necessary to obtain an airway anti-inflammatory effect. On the contrary, inhibition of the allergen-induced accumulation of fluid in the lung, via activation of the $S1P_1$ receptor, is obtainable without systemic effects.
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Abbreviations: BAL, broncho-alveolar lavage; MRI, magnetic resonance imaging; OVA, ovalbumin; S1P, sphingosine-1-phosphate

Introduction

Inflammatory processes represent a protective response against insults to the organism. They involve the recruitment of many cell types and the production of various inflammatory mediators in attempts to contain and reverse the insult. However, chronic inflammation can lead to irreversible tissue destruction. There is now evidence that sphingosine-1-phosphate (S1P) and its receptors regulate inflammatory processes on several levels. S1P is primarily generated from the cell membrane constituent sphingomyelin via the metabolites ceramide and sphingosine. A variety of inflammatory factors, including interleukin-1, tumour necrosis factor-α and vascular endothelial growth factor, activate sphingosine kinase-1 to convert pro-apoptotic sphingosine into anti-apoptotic S1P (Chalfant and Spiegel, 2005) and reduce the turnover and death of pro-inflammatory cells, for example of effector T cells (Goetzl *et al.*, 1999). Furthermore, S1P regulates cell migration and trafficking either by acting as a chemoattractant for migrating cells, or by modulating permeability barriers between different tissues (Brinkmann, 2007).

The S1P receptor agonist, FTY720, is a novel immunomodulator that sequesters lymphocytes into secondary lymphoid organs, and thereby prevents their migration to sites of inflammation (Brinkmann, 2007). Acting through four of the five G protein-coupled S1P receptors ($S1P_1$, $S1P_{3–5}$), with nanomolar potency (Pan *et al.*, 2006), FTY720 is able to modulate cell trafficking and inflammation, as well as endothelial and epithelial barrier function by variable means, depending on the S1P receptor type involved and the targeted cell type/tissue. We have shown that oral treatment with FTY720 inhibits the ovalbumin (OVA)-induced lung inflammation in a mouse model of asthma (Sawicka *et al.*, 2003). More recently, it was shown that intratracheal FTY720 suppresses T helper

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type 2-dependent mouse eosinophilic airway inflammation and bronchial hyperresponsiveness without causing lymphopenia and T cell retention in the lymph nodes but rather by inhibiting the migration of lung dendritic cells to the lung lymph nodes (Idzko *et al.*, 2006). However, in both studies, the S1P receptor responsible for this effect was not characterized.

We have recently described, in an *in vivo* murine model of allergen-induced inflammation, the use of proton magnetic resonance imaging (MRI) to non-invasively assess the lung plasma leakage- and mucus-related signals, which correlated with inflammatory parameters recovered from the bronchoalveolar lavage (BAL) and with histological analyses (Ble *et al.*, 2008).

In this work we have used this model to investigate the effects of intranasal administration of FTY720 prior to allergen challenges of actively sensitized mice. Budesonide, an inhaled steroid available for the treatment of asthma, was used as a reference compound. Furthermore, the targeted S1P receptors were investigated using S1P$_3$-deficient mice and AUY954, a S1P$_1$ receptor-selective agonist with nanomolar potency and more than 300-fold selectivity over the other S1P receptors (Pan *et al.*, 2006).

Methods

Animals

Seven- to 9-week-old C57BL/6 female mice (Janvier, Le Genest-St-Isle, France) were used throughout the study. Naïve wild-type or S1P$_3$-deficient mice (Ishii *et al.*, 2001) were bred in house. All animals were housed in a temperature- and humidity-controlled environment, with free access to standard mouse chow and tap water. Experiments were carried out with the approval of the Veterinary Authority of the City of Basel (licence number 1989).

Allergen sensitization and challenge

Ovalbumin (OVA) (250 μg·mL^{-1}) was mixed (30 min on ice) in a blender with aluminium hydroxide (10 mg·mL^{-1}) in saline and injected intraperitoneally on days 0 and 14 (0.2 mL per animal). On day 18 mice were lightly anaesthetized with 2% isoflurane, and 100 μg of OVA in 25 μL of saline or vehicle (25 μL of saline) was administered intranasally via a micropipette (12.5 μL per nostril). Animals were allowed to recover immediately after. This procedure was repeated on days 19, 20 and 21.

Drug treatment

Budesonide and the selective S1P$_1$ receptor agonist AUY954 were dissolved in dimethyl sulphoxide (2% final concentration) and diluted with saline. FTY720 was dissolved in distilled water. Animals were lightly anaesthetized with 2% isoflurane and budesonide (3 mg·kg^{-1}), FTY720 (3, 10, 30 or 100 μg·kg^{-1}), AUY954 (3 or 10 μg·kg^{-1}) or their corresponding vehicles were administered intranasally, in a volume of 25 μL (12.5 μL per nostril), 30 min before each intranasal OVA challenge.

Magnetic resonance imaging

At baseline (on day 16) and 24 h after the last OVA challenge, measurements were carried out *in vivo* with a Biospec 47/40 spectrometer operating at 4.7 T, equipped with an actively shielded gradient system capable of generating a gradient of 200 mT·m^{-1}. A birdcage resonator of 32 mm diameter was used for excitation and detection. Gradient-echo images were acquired with the following parameters: repetition time 5.6 ms, echo time 3.5 ms, band width 100 kHz, flip angle of the excitation pulse approximately 10°, matrix size 256 × 128, slice thickness 0.75 mm and field of view 3 × 3 cm^2. A single slice image (acquisition time of 74 s) was obtained by computing the two-dimensional Fourier transform of the averaged signal from 60 individual acquisitions and interpolating the data set to 256 × 256 pixels. The entire lung was covered by 16 consecutive transverse slices. During measurements, mice were anaesthetized with 1.5% isoflurane in a mixture of O_2/N_2O (1:2), administered via a face cone. Animals were allowed to breathe spontaneously, and neither respiratory nor cardiac gating was applied. Mice were kept warm by means of a flow of warm air (37 ± 1°C).

Image analysis

The volume of MRI signals was quantified using a semi-automatic segmentation procedure implemented in the IDL (Interactive Data Language Research Systems, Boulder, CO, USA) environment on a Linux system (Beckmann *et al.*, 2001). Images were first weakly lowpass filtered with a Gaussian profile filter and then transformed into a set of four grey level classes using adaptive Lloyd-Max histogram quantification. The highest grey level class in the transformed images could be extracted interactively by use of a region grower. Contour serving as a growing border was drawn to control region growing manually. Because the signals from extravascular fluid and vessels were of comparable high intensities, the volume corresponding to the vessels was assessed on baseline images and then subtracted from the volumes determined on post-challenge images.

Invasive assessment of inflammatory parameters

Immediately after MRI acquisition, mice were maintained under anaesthesia (isoflurane 3% v/v), and 500 μL of blood was sampled by retro-orbital bleeding in ethylenediaminetetraacetic acid (EDTA)-coated Eppendorf tubes and subjected to haematological analysis. Absolute and differential leukocyte counts, including lymphocyte counts, were obtained with a Technicon H1-E analyzer. After blood sampling, mice were killed by an i.p. injection of 0.2 mL of pentobarbitone (250 mg·kg^{-1}). The trachea was cannulated and the lung washed out by gently injecting eight times via the trachea, without any chest massage, 0.5 mL of a BAL solution prepared with: (i) Hank's balanced salt solution; (ii) EDTA, 1 mM; (iii) 4-(2-hydroxyethyl)-1-piperazinethanesulphonic acid, 0.1 M; (iv) D-glucose anhydrous, 1 g; and (v) distilled water. An aliquot from BAL fluid was used for total cell count in an automatic cytometer. From each aliquot, a sample containing approximately 50 000 cells was used for cytospin preparation. Cells were stained with Diff-Quik and a differential count of

200 cells (eosinophils, macrophages, neutrophils, lymphocytes) was obtained by observing cytospin slides under a classic light microscope (×100, oil immersion) using standard morphological criteria.

Data analysis

Results are expressed as means ± SEM. Statistical comparisons were performed using an ANOVA with Bonferroni correction for multiple comparisons, and a *P*-value of less than 0.05 was considered significant (Systat V.10.2).

Drugs and materials

Ovalbumin was obtained from Fluka (Buchs, Switzerland); isoflurane from Abbott (Cham, Switzerland); budesonide from Sicor (Milan, Italy); AUY954 and FTY720 from Novartis AG (Basel, Switzerland). The Biospec 47/40 spectrometer was from Bruker Medical Systems (Karlsruhe, Germany); the automatic cytometer from Sysmex (Norderstedt, Germany) and the Technicon H1-E analyzer from Bayer Diagnostics (Zurich, Switzerland).

Results

Effect of intranasal budesonide pretreatment on allergen-induced lung inflammation and MRI fluid signals

To verify whether anti-inflammatory treatment could affect airways fluid signals detected by MRI, the effect of budesonide (3 $mg \cdot kg^{-1}$), an inhaled glucocorticosteroid widely used to treat human asthma, was tested in the model. Figure 1 shows

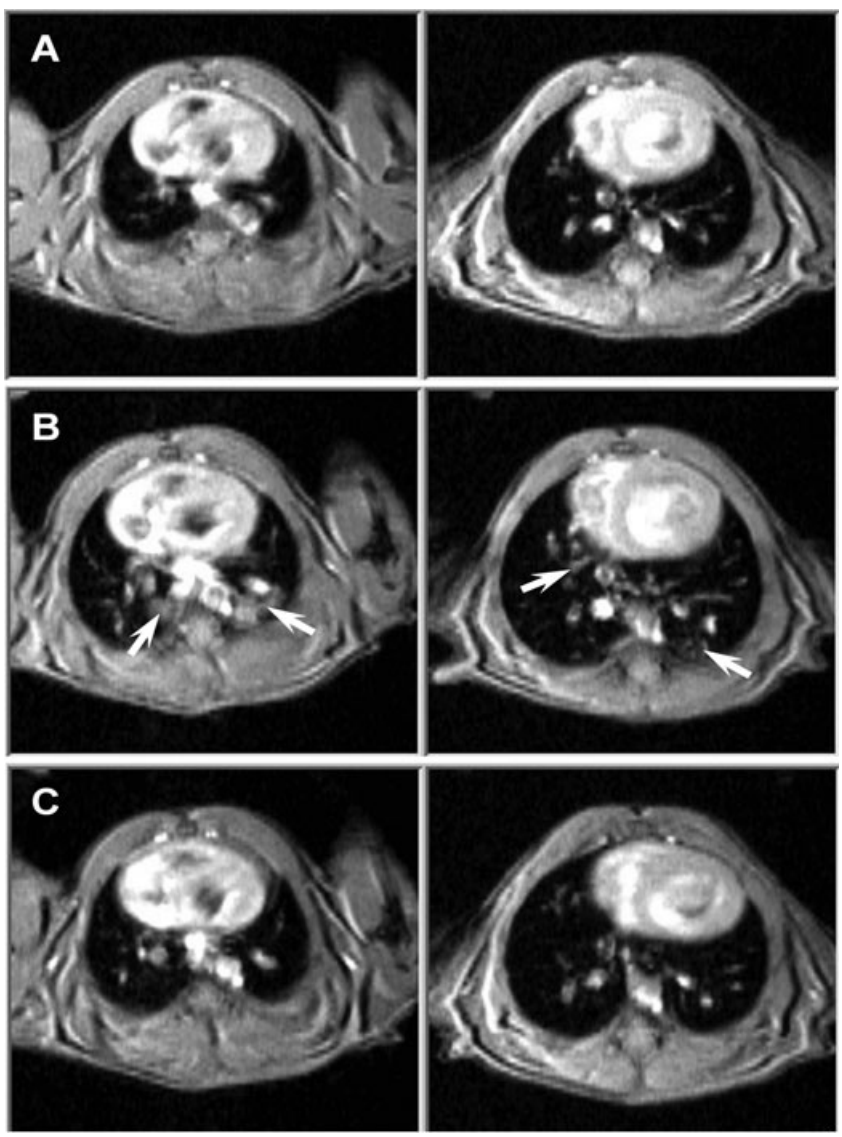

Figure 1 Representative magnetic resonance imaging (MRI) transverse sections through the thorax of actively sensitized mice, obtained 24 h after the last intranasal challenge. (A) Saline-challenged mice treated with vehicle (2% dimethyl sulphoxide in saline). (B) Ovalbumin-challenged mice treated with vehicle. (C) Ovalbumin-challenged mice treated with budesonide (3 $mg \cdot kg^{-1}$). Arrows indicate MRI lung fluid signals.

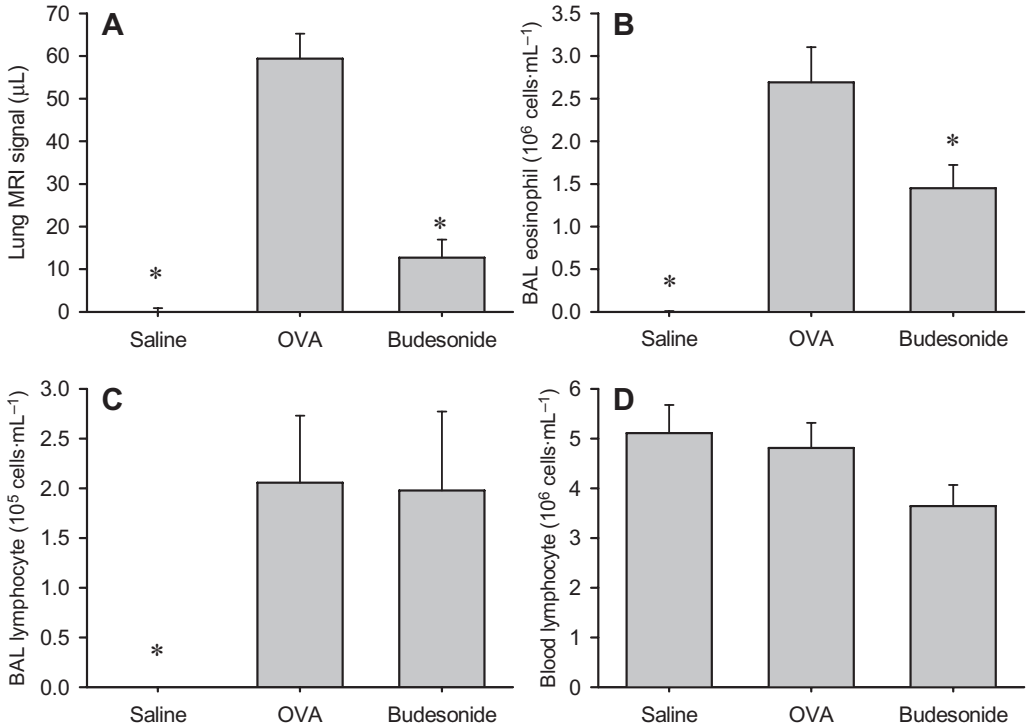

Figure 2 Effects of budesonide on the allergen-induced (A) lung magnetic resonance imaging (MRI) fluid signals, numbers of (B) eosinophils and (C) lymphocytes in the broncho-alveolar lavage (BAL) fluid and (D) blood lymphocytes. Control mice were intranasally treated, 1 h before each challenge, with vehicle (2% dimethyl sulphoxide in saline) and either challenged with saline (saline, $n = 8$) or ovalbumin (OVA, $n = 8$). Budesonide, 3 mg·kg^{-1}, was given intranasally 1 h before each OVA challenge (budesonide, $n = 8$). The asterisk indicates significantly different from the saline-treated, ovalbumin-challenged mice.

representative magnetic resonance images obtained 24 h after the last OVA or saline challenges. No fluid signals were present in the lungs of saline-challenged animals (Figure 1A). Fluid signals were apparent in the lungs of vehicle-treated, OVA-challenged mice (Figure 1B), and these were reduced after intranasal treatment with budesonide (Figure 1C).

Quantification of the lung MRI signals showed that, compared with vehicle treatment, budesonide led to an approximately 80% reduction of the fluid signals (Figure 2A). The reduced number of infiltrated BAL eosinophils (Figure 2B) confirmed the local anti-inflammatory effectiveness of the corticosteroid. Budesonide had no effect on the number of BAL or blood lymphocytes (Figure 2C and D, respectively).

Effect of intranasal FTY720 pretreatment on allergen-induced lung inflammation

At doses from 3 to 100 µg·kg^{-1}, FTY720, administered intranasally prior to each OVA challenge, reduced by approximately 50% the lung MRI signals (Figure 3A). However, FTY720 was only able to significantly decrease the number of BAL eosinophils at the highest dose used (100 µg·kg^{-1}) (Figure 3B), but this dose did not significantly affect the BAL lymphocyte numbers (Figure 3C). In addition, the compound dose-dependently diminished the blood lymphocytes with a significant inhibition observed from the dose of 30 µg·kg^{-1} (Figure 3D).

Effect of intranasal pretreatment with FTY720 on allergen-induced lung inflammation in S1P$_3$-deficient mice

To determine whether S1P$_3$ receptors were implicated in the effects described above, we compared the effects of 100 µg·kg^{-1} FTY720 in allergen-challenged wild-type mice with those in S1P$_3$-deficient mice. The effects of FTY720 on the OVA-induced increase in lung MRI signal (Figure 4A), BAL eosinophil (Figure 4B) and lymphocyte (Figure 4C) infiltration and blood lymphocyte counts (Figure 4D) were comparable in both wild-type and S1P$_3$-deficient mice.

Effect of intranasal selective S1P$_1$ agonist: AUY954, on allergen-induced lung inflammation in C57BL/6 mice

Intranasal administration of the S1P$_1$ receptor-selective agonist, AUY954, at doses of 3 and 10 µg·kg^{-1}, prior to OVA challenges reduced the MRI lung fluid signal in response to allergen by about 50% of that observed in the vehicle-treated mouse (Figure 5A). At both doses, AUY954 significantly inhibited the number of BAL lymphocytes (Figure 5C) but had no effect on the BAL eosinophils (Figure 5B) or on the blood lymphocytes (Figure 5D).

Discussion

It has been recently shown that MRI provides a relevant means to assess lung fluid signals associated with airway

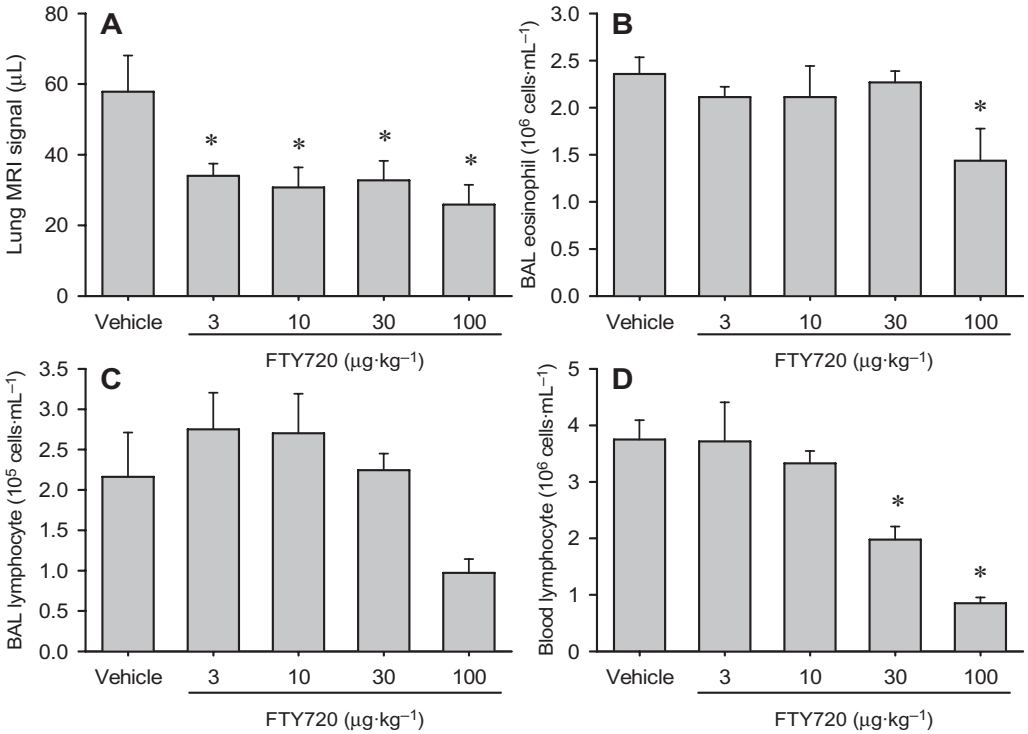

Figure 3 Effects of FTY720 on the allergen-induced (A) lung magnetic resonance imaging (MRI) fluid signals, numbers of (B) eosinophils and (C) lymphocytes in the broncho-alveolar lavage (BAL) fluid and (D) blood lymphocytes. Control mice were intranasally treated, 1 h before each ovalbumin challenge, with vehicle (saline) (vehicle, $n = 8$). FTY720, 3–100 $\mu g \cdot kg^{-1}$, was given intranasally 1 h before each ovalbumin challenge ($n = 6$–8 per dose). The asterisk indicates significantly different from the saline-treated, ovalbumin-challenged mice (vehicle control mice).

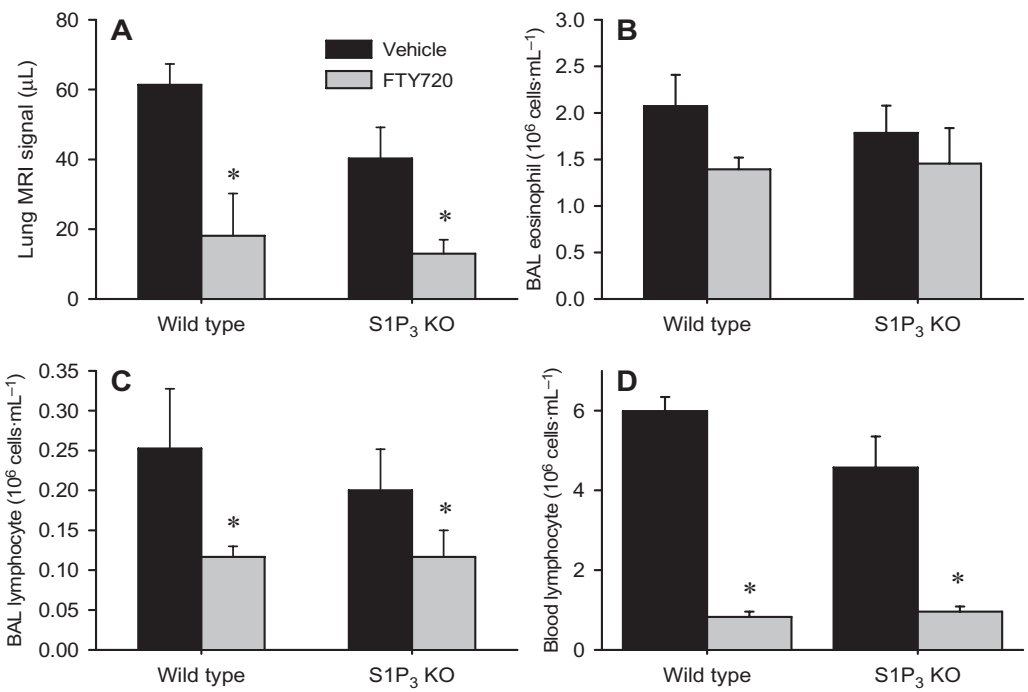

Figure 4 Effects of FTY720 on the allergen-induced (A) lung magnetic resonance imaging (MRI) fluid signals, numbers of (B) eosinophils and (C) lymphocytes in the broncho-alveolar lavage (BAL) fluid and (D) blood lymphocytes in $S1P_3$-deficient mice and their wild-type littermates. Control mice were intranasally treated with vehicle (saline) 1 h before being challenged with ovalbumin ($n = 6$). FTY720, 100 $\mu g \cdot kg^{-1}$, was given intranasally 1 h before each ovalbumin challenge ($n = 6$). Statistical significance, indicated by the asterisk, indicates significantly different from saline-treated, ovalbumin-challenged mice (vehicle control mice).

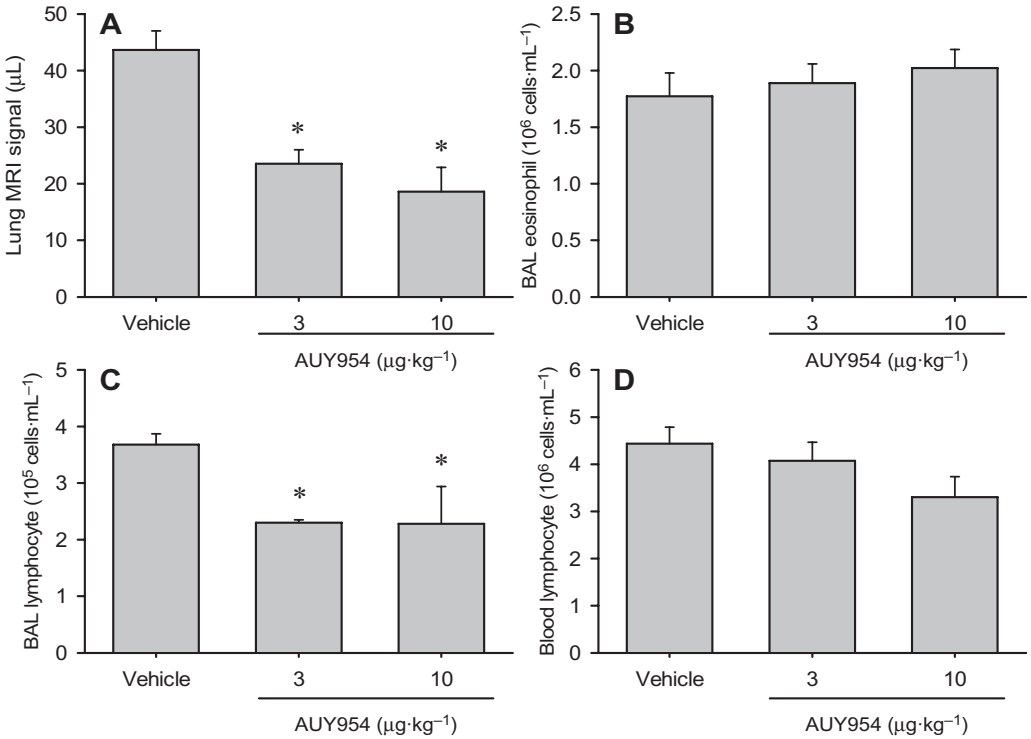

Figure 5 Effects of AUY954 on the allergen-induced (A) lung magnetic resonance imaging (MRI) fluid signals, numbers of (B) eosinophils and (C) lymphocytes in the broncho-alveolar lavage (BAL) fluid and (D) blood lymphocytes. Control mice were intranasally treated, 1 h before each ovalbumin challenge, with vehicle (2% dimethyl sulphoxide in saline) (vehicle, $n = 8$). AUY954, 3 and 10 μg·kg^{-1}, was given intranasally 1 h before each ovalbumin challenge ($n = 8$ per dose). The asterisk indicates significantly different from saline-treated, ovalbumin-challenged mice (vehicle control mice).

inflammation induced in mice by allergen challenge (Ble *et al.*, 2008). Using this model, in the present work we have demonstrated that FTY720, given locally to the airways and acting on the S1P$_1$ receptor, is a potent inhibitor of allergen-induced lung MRI fluid signal accumulation at doses that have no effect on airway cellular inflammation or blood lymphocyte numbers.

In confirmation with our previous findings (Ble *et al.*, 2008), repeated instillation of OVA resulted in intense MRI signals in the lungs of allergen-sensitized mice. In the present work we have shown that these signals can be significantly reduced by pretreatment with a standard anti-inflammatory agent, budesonide. The inhibition of the allergen-induced increase in the lung MRI signals with budesonide treatment was concomitant with a reduction of BAL eosinophil infiltration. These data obtained in the mouse are analogous to our results obtained in a rat model of allergen-induced pulmonary inflammation (Tigani *et al.*, 2002; 2003), indicating that in the mouse, the effects of anti-inflammatory drugs are also non-invasively quantifiable by MRI.

We have previously shown that FTY720 is an effective oral drug for treatment of experimental asthma induced in the mouse (Sawicka *et al.*, 2003). In this study the systemic immunodeficiency caused by S1P receptor-mediated inhibition of lymphocyte egress from the lymph nodes was probably responsible for the observed reduction of the inflammatory response to allergen. More recently, with the aim of minimizing systemic effects, Idzko *et al.* (2006) investigated the effect of intratracheal administration of FTY720 in a murine model of allergic airway inflammation and were able to suppress Th2-related eosinophilic airway inflammation without causing lymphopenia. The authors postulated that the effectiveness of local treatment was achieved by inhibition of the lung dendritic cells capacity to interact with allergen-specific Th2 cells in lymph nodes. Contrasting with these observations, our data suggested that, when applied intranasally, FTY720 reduced eosinophilic airway inflammation only when the levels of circulating lymphocytes were decreased. This discrepancy might be explained by the different routes of administration (intranasal vs. intratracheal) and/or mouse strains employed.

In the present study, we showed that, even at doses that did not induce a decrease in the number of blood lymphocytes, FTY720 potently decreased the volume of lung MRI signals detected in response to allergen challenge. Because MRI fluid signals detected in this murine model reflect both plasma leakage and mucus secretion (Ble *et al.*, 2008), it is plausible that low doses of FTY720 could have affected the integrity of the lung barrier. Indeed, it has been shown that S1P receptors can differentially modulate the permeability of the lung barrier and plasma exudation (Sanchez *et al.*, 2003; Peng *et al.*, 2004; McVerry and Garcia, 2005). On the one hand, activation of the S1P$_2$ receptor on the endothelium increases vascular permeability (Sanchez *et al.*, 2007). Similarly, activation of the airway epithelium S1P$_3$ receptor increases the epithelial permeability (Gon *et al.*, 2005). On the other hand, activation of the endothelial S1P$_1$ receptor has been demonstrated to inhibit vascular leakage *in vivo* (Sanna *et al.*, 2006). As FTY720

is unable to activate the $S1P_2$ receptor (Pan *et al.*, 2006), the implication of this receptor subtype in our study can be excluded.

The fact that FTY720-induced inhibition of the lung MRI signals is not affected in $S1P_3$-deficient mice suggests that, in agreement with the known role for this receptor, this phenomenon is not mediated via the $S1P_3$ receptor. Nevertheless, deletion of the $S1P_3$ receptor abolishes the transient disturbance of the integrity of epithelial tight junctions induced by local exogenous $S1P_3$ receptor agonists (Gon *et al.*, 2005). Interestingly, in our study the $S1P_3$ deletion led to a small (albeit not significant) reduction of MRI fluid signals induced by allergen provocation in vehicle-pretreated animals. This suggests that endogenous S1P, produced in response to multiple allergen challenges, can contribute to the increased lung MRI fluid signals via activation of the $S1P_3$ receptor.

Similar to that observed for FTY720, the selective $S1P_1$ agonist AUY954 potently reduced allergen-induced MRI signals without having a marked anti-inflammatory effect on the cellular infiltration into the airways. This strongly suggests that the inhibition of the MRI signal observed with FTY720 is mediated by $S1P_1$ receptors, probably by inhibiting plasma leakage at the level of the endothelium (Sanna *et al.*, 2006). At the doses used (1 and 10 $\mu g \cdot kg^{-1}$), AUY954 did not influence the number of BAL eosinophils or the number of circulating lymphocytes. This probably reflects a low systemic availability of the compound when given locally to the airways. Unfortunately, because of its poor solubility, AUY954 could not be given at higher doses.

In the present study, we have shown the usefulness of lung MRI for investigating airway inflammation and its pharmacology. By evaluating the effects of intranasal administration of FTY720 prior to allergen challenge in actively sensitized mice, we have obtained results that suggest the reduction of lung fluid MRI signals, which are related to plasma leakage and mucus secretion, is mainly explained by an action on endothelial barrier integrity through $S1P_1$ receptors, rather than by a local and/or systemic anti-inflammatory effect of the compound.

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