Molecular Pharmacology Fast Forward. Published on October 8, 2010 as doi:10.1124/mol.110.066811

MOLPHARM/2010/066811

Modulation of chemokines and allergic airway inflammation by selective local S1P1 agonism in lungs

David Marsolais, Saiko Yagi, Tomoyuki Kago, Nora Leaf and Hugh Rosen

Department of Chemical Physiology (DM, SY, TK, NL, HR) and Molecular Screening Center (HR), The Scripps Research Institute, La Jolla, CA, USA.

Current addresses are Kyorin Pharmaceutical Co. Ltd, Nogi, JAPAN (SY, TK); Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec, Université Laval, Québec, Canada (DM).

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

MOLPHARM/2010/066811

Running title: S1P1 Alleviates Allergic Airway Inflammation

Corresponding author and to whom reprint requests and page proofs should be addressed:

Hugh Rosen,
MEM L55, TSRI,
10550 N. Torrey Pines Rd,
La Jolla, CA 92037.
Phone: (858) 784-2397;
Fax: (858) 784-2988
hrosen@scripps.edu

Number of text pages (including references) :	34
Number of tables :	0
Number of figures :	6
Number of references :	39
Number of words in abstract :	199
Number of words in introduction :	582
Number of words in discussion :	1377

List of nonstandard abbreviations: AAI, allergic airway inflammation; AAL-R, (R)-2-amino-4- (4-heptyloxyphenyl)-2-methylbutanol; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow-derived dendritic cells; CFSE, carboxy- fluorescein diacetate, succinimidyl ester; CTR, control; CD, cluster of differentiation; cPLA2 cytosolic Phospholipase A2; DC, dendritic cell; ILN, inguinal lymph nodes; MLN, mediastinal lymph nodes; PBS, phosphate buffered saline; OVA, ovalbumin; RANTES, Regulated upon Activation, Normal T-cell Expressed, and Secreted; S1P, Sphingosine-1-phosphate; S1P1-5, Sphingosine-1-phosphate receptors 1 to 5; SK, sphingosine kinase; TARC, Thymus- and Activation-Regulated Chemokine; VEH, vehicle;

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

MOLPHARM/2010/066811

Abstract

Sphingosine-1-phosphate and its receptors have emerged as important modulators of the immune response. The sphingosine-1-phosphate pro-drug FTY720 can alleviate experimental allergic airway inflammation. Nevertheless, the role of individual sphingosine-1-phosphate receptors in the regulation of allergic airway inflammation remains undefined. Using a newly characterized potent and selective sphingosine-1-phosphate receptor 1 (S1P1) agonist with physical properties allowing airway delivery, we studied to the contribution of S1P1 signaling to eosinophilic airway inflammation induced in ovalbumin-immunized mice by airway challenges with ovalbumin. Airways delivery of receptor-non-selective sphingosine-1-phosphate pro-drug significantly inhibits the sequential accumulation of antigen presenting dendritic cells and $CD4^+$ T cells in draining lymph nodes. This in turn suppressed by >80% the accumulation of $CD4^+$ T cell and eosinophils in the airways. Systemic delivery of sphingosine-1-phosphate pro-drug or of an S1P1-specific agonist at doses sufficient to induce lymphopenia did not inhibit eosinophil accumulation in the airways. In contrast, local airway delivery of S1P1-specific agonist inhibited airways release of endogenous CCL5 and CCL17 chemokines, and significantly suppressed accumulation of activated T cells and eosinophils in the lungs. Specific S1P1 agonism in lungs contributes significantly to anti-inflammatory activities of sphingosine-1-phosphate therapeutics by suppressing chemokine release in the airways, and may be of clinical relevance.

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Sphingosine-1-phosphate (S1P) is produced by phosphorylation of sphingosine by sphingosine kinases (SK1 and SK2). Along with its 5 high affinity G-protein-coupled receptors (S1P1 to S1P5), S1P has emerged as significant modulator of processes underlying pathogenesis of asthma. For instance, SK1-derived S1P regulates pro-inflammatory signaling pathways, including activation of NF-Kappa B (Alvarez et al.). S1P1 regulates endothelial barrier integrity (Sanna et al., 2006), cytokine and adhesion molecules expression (Lien et al., 2006), lymphocyte maturation, differentiation and trafficking (Liu et al., 2009; Sanna et al., 2004), as well as mast cell migration (Jolly et al., 2004). S1P2 has been involved in the regulation of mast cell degranulation (Jolly et al., 2004) and tissue remodeling (Skoura et al., 2007) while S1P3 was shown to modulate dendritic cell trafficking (Niessen et al., 2008). In addition, SK1 and SK2 (Liu et al., 2000), as well as receptors S1P1 to S1P4 are expressed in the lung tissue (Graler et al., 1998; Zhang et al., 1999). Importantly, S1P levels are increased in the airways of asthmatic patients (Ammit et al., 2001) but whether or not this increase is deleterious or protective remains unclear.

The role of S1P and its receptors in the pathogenesis of allergic airway inflammation remains controversial. For instance, the S1P pro-drug FTY720 alleviates the salient features of allergic airway inflammation (AAI) including accumulation of eosinophils and T cells in the airways and development of bronchial hyperresponsiveness (Idzko et al., 2006; Sawicka et al., 2003). In

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

contrast, administration of exogenous S1P exacerbates bronchial hyperresponsiveness (Roviezzo et al., 2004) by favoring mast cell and eosinophil accumulation in the lung, while reduction of S1P levels using sphingosine kinases inhibitors leads to alleviation of AAI (Nishiuma et al., 2008).

Non-selective S1P receptor agonists, with significant additional off-target activities, limit mechanistic understanding of the system. Indeed, sphingosine analogs like FTY720 or AAL-R interact with or inhibit multiple proteins including sphingosine kinases, S1P lyase, cPLA2, lipid phosphatases and, after phosphorylation, 4 of the 5 S1P receptors (S1P1, S1P3, S1P4 and S1P5) (reviewed in (Marsolais and Rosen, 2009)). Similarly, modulation of AAI by genetic or chemical inhibition of sphingosine kinase activities can either result from the build up of upstream metabolites in the metabolic pathway leading to S1P generation (Billich et al., 2003; Petrache et al., 2005), or potentially, from decreased stimulation of any of the 5 S1P receptors. Thus, current genetic systems and receptor-subtype non-selective chemical probes are limited in the analysis of the complex sphingosine -S1P -S1P receptor signaling system in vivo.

S1P1 agonism shows therapeutic potential for alleviation of pulmonary immunopathology since it regulates T cell trafficking (Brinkmann et al., 2004; Sanna et al., 2004), barrier integrity (Marsolais and Rosen, 2009), and cytokine release (Lien et al., 2006). Genetic deletion of S1P1 did not clarify the role of this

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

receptor in the regulation of pulmonary immune response, as it is embryonic lethal. Moreover, existing S1P1 agonists like SEW2871 or AUY954 (Pan et al., 2006) had poor water solubility, which limited their use in the airways. Indeed, these agonists need to be dissolved in common solvents including DMSO or methanol, which are incompatible with lung delivery (Massion et al., 1996). Usage of receptor-specific agonists with physical properties allowing airway delivery is therefore a preferred experimental strategy to dissect site- and subtype- specific receptor contributions in the pathogenesis of respiratory diseases. Here we show that airway delivery of a water-soluble S1P1-selective agonist dampens recruitment of activated CD4+ cells and inhibits eosinophilic airway inflammation by regulating the release of chemokines in the airways.

Materials and Methods

Mice

Male C57BL/6j and BALB/c were maintained in a closed breeding facility at The Scripps Research Institute. The handling of all mice conformed to the requirements of the National Institutes of Health and The Scripps Research Institute animal research committee.

Allergic airway inflammation model (Fig 1)

Male mice (6-8 weeks old) were immunized intraperitoneally (i.p.) on day 0 and 7 with 10 µg of ovalbumin (OVA) (Grade V; Sigma-Aldrich) in 0.1 ml of aluminum hydroxide gel (Sigma) or 0.1ml of aluminum hydroxide gel with saline for control.

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Recall response was induced on days 19 to 21 or 23 with intratracheal (i.t.) delivery, once daily, of PBS containing 0.1% OVA (Sigma, grade V).

Compounds

AUY954 (synthesized according to published methods, Pan et al, 2006) was dissolved in polyethylene glycol 300 and 5% dextrose while AAL-R and the tartrate salt of CYM-5442 ([2-(4-(5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1*H*inden-1-yl amino)) were dissolved in water. AUY954 was administered by gavage while AAL-R ((*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol) and CYM-5442 were either delivered i.p or i.t. (local treatment) Mice were anesthetized with isoflurane for i.t. delivery of compounds.

Flow cytometric analyses

Single cell suspensions were obtained from bronchoalveolar lavage fluid (BALF), whole lungs or lymph nodes as described earlier (Marsolais et al., 2008). The frequencies of dendritic cells, T cells and eosinophils were assessed by flow cytometry. To do so, single cell suspensions were processed for surface staining with fluorochrome-labeled antibodies raised against murine CD3e, CD4, CD8a, CD11b, CD11c, CD45.2, CD44, CD62L, GR-1, F4/80 and CCR-3 (BD Pharmingen and eBioscience). Total number of viable cells was evaluated by the trypan-blue exclusion method and multiplied by frequencies obtained by flow cytometry to compute the absolute number of specific cell subsets.

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Cytokine quantification

BALF were performed with 1ml of PBS. Samples were kept on ice and cell-free supernatant was harvested after centrifugation at 4°C. A cocktail of protease inhibitors (Complete, Roche diagnostics) was added to the samples which were stored at -20°C until multiplex ELISA analysis (Quansys Biosciences) (Marsolais et al., 2009).

In vivo uptake of OVA- Alexa Fluor 647

Mice were administered by gavage with either 3 mg/kg of AUY954 or vehicle (polyethylene glycol 300, 5% dextrose). Three hours later, 100 µg of OVA- Alexa Fluor 647 (Invitrogen) in 50 µl of PBS or 50 µl of PBS was administered i.t. under isoflurane anesthesia. Twenty-four hours after injection, Alexa Fluor 647^+ $CD11C^+$ DCs were quantified in lungs and mediastinal lymph nodes

Bone marrow-derived DCs in vivo migration assay

DCs were derived from bone marrow cells (BMDCs) as described previously (Lutz et al., 1999). Cultured DCs were incubated with DMSO or 1 µM AUY954. Thirty minutes later, 100 µg/ml of OVA was added into the DCs culture and incubated overnight. DCs were then washed and labeled with carboxy-fluorescein diacetate, succinimidyl ester (CFSE) (Invitrogen). 2 x 10^6 CFSE-labeled DCs were transferred i.t. into congenic mice. The number of $CFSE^+$ $CD11c^+$ cells was quantified in lungs and mediastinal LNs 48 hours after DC transfer.

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Statistical Analysis

Bars represent means of a group of mice ± standard error of the mean. Averages between groups were compared using one-way analysis of variance (with Tukey-Kramer post hoc test); or with two-sided unpaired Student's *t* test. When appropriate, logarithmic transformation was applied on the data to insure stability of variance between groups. For analyses of variance, labeling with a same alphabetic letter represents absence of differences between groups. Asterisk (*) was used to mark significant differences between two groups when T tests were performed. The results were considered significant with p-values ≤ 0.05. Three to six mice were used in each individual group included in an experiment; and a minimum of 2 experiments was performed for each set of data presented in figures. Data were analyzed using the statistical package program STATA vX.X.

Results

Non-selective S1P receptor agonist pro-drug alleviates AAI

In a first series of experiments, we determined how local i.t. delivery of the S1P receptor agonist pro-drug AAL-R modulated the immune response during the recall response of AAI. As in non-immunized mice (CTR), mice immunized with saline and challenged with OVA (Saline/OVA mice; Fig 1) did not display salient features of allergic airway inflammation (AAI), i.e. massive accumulation of T cells and eosinophils in BALF (Fig 2A&B). As expected, significant

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

accumulation of dendritic cells (DC) and T cells in draining mediastinal lymph nodes (Fig 2C&D), as well as infiltration of $CD4^+$ T cells and eosinophils into the airways (Fig 2A&B) were observed in mice sensitized and then challenged with OVA (OVA/OVA mice).

Local i.t. delivery of 0.1 mg/kg of the S1P pro-drug AAL-R during the recall phase of AAI inhibited the accumulation of $CD11c^+$ DC in the draining mediastinal lymph nodes (Fig 2C) of OVA/OVA mice, leading to impaired expansion of $CD4^+$ T cells in mediastinal lymph nodes (Fig. 2D) and to >70% inhibition of $CD4^+$ T cell accumulation in the BALF (Fig 2A). Moreover, the accumulation of eosinophils in BALF was reduced by 80%, when compared to VEH-treated OVA/OVA mice, 24 h following the last OVA i.t. challenge (Fig 2B). These results confirm alteration of DC trafficking from lungs to lymph nodes as a primary immunosuppressive mechanism of airway-delivered sphingosine analogs (Idzko et al., 2006; Marsolais et al., 2008; Marsolais et al., 2009).

Contrasting the effects of systemic delivery of pro-drug or selective S1P1 agonist on AAI

To quantify the contribution of systemic effects of S1P receptor activation in the recall phase of AAI, mice were gavaged with either AAL-R or with the S1P1-selective agonist AUY954 (Pan et al., 2006), at doses sufficient to induce sustained lymphopenia ((Marsolais et al., 2008; Pan et al., 2006); data not shown). Systemic delivery of either AAL-R or AUY954 failed to significantly inhibit accumulation of $CD11c^+$ DCs in mediastinal lymph nodes of OVA/OVA mice 24h

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

following the last OVA challenge, compared to OVA/OVA mice treated with vehicle (VEH) (Fig 3A). As expected by lymphocyte sequestration from peripheral blood, systemic delivery of these drugs mildly altered $CD4^+$ T cell content of mediastinal lymph nodes (Fig 3B), reduced the number of transiting $CD4^+$ T cells in the lungs (Fig 3C), but failed to significantly alter the accumulation of eosinophils in BALF (Fig 3D). Systemic delivery of S1P prodrug increased the number of total T cells in non-draining inguinal popliteal lymph nodes (not shown) through sequestration. Thus, systemic delivery both S1P pro-drug or S1P1-specific agonist after allergen challenge, at doses sufficient to induce lymphopenia, fails to replicate the effects of airway-delivered agonist, and uncouples pulmonary effects from the alteration of trafficking of naïve lymphocytes.

S1P1 receptor activation does not interfere with antigen transport from lungs to mediastinal lymph nodes.

Given that the number of $CD4^+$ T cells was reduced in BALF by local i.t. S1P pro-drug treatment, we tested the hypothesis that S1P1 activation short circuited DC trafficking from lungs to mediastinal lymph nodes, as previously shown with the broad S1P receptor agonist prodrugs (Figure 2; (Idzko et al., 2006),(Marsolais et al., 2009) and unpublished observations). Systemic activation of S1P1 can increase the number of DCs in the blood (Lan et al., 2005). However, whether or not this effect is mediated through direct activation of S1P1 on DCs remains unknown. In agreement with results shown in figure 3A,

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

oral delivery of AUY954 did not alter the movement of $CD11c^+$ carrying Alexa Fluor 647-coupled OVA from lungs to mediastinal lymph nodes (Fig 4A). In addition, in vitro treatment of CFSE-stained OVA-treated BMDCs with micromolar concentration of AUY954 did not impair the ability of DCs to migrate to the mediastinal lymph nodes (Fig 4B), which resulted in a normal T cell response in the BALF (Fig 4C). In addition, S1P3-null DCs pulsed with OVA induced AAI (not shown). Together, these results suggest that the mechanism of S1P1-mediated inhibition of T cell accumulation in BALF during AAI is independent of both migration and antigen presentation by DC.

S1P1 activation in lungs inhibits $CD4^+$ T cell and eosinophilic responses.

Since S1P1 activation did not alter DC function in the airways, we tested if this receptor was dispensable for local inhibition of AAI. Selective airway modulation of S1P1 was made possible by the recent development of a water-soluble tartaric salt of the S1P1-selective receptor agonist (CYM5442 (Gonzalez-Cabrera et al., 2008)). Surprisingly, daily local i.t. delivery of CYM-5442 during the recall response of AAI strongly inhibited the T cell and eosinophilic responses in OVA/OVA mice when compared to the VEH group (Fig 5A&B). Local i.t. treatment with CYM-5442 inhibited the accumulation of effector/memory $CD44^{hi}$ $CD4^+$ T cells subsets (Fig 5C) in the BALF, when compared to the VEH group. On the other hand, i.p. delivery of CYM-5442 was inefficient at inhibiting the accumulation of eosinophils, total and effector/memory T cells in BALF (Fig 5), when compared to the local i.t. delivery route. Along with the DC data presented

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

in figure 4, these data (Fig. 5) support a mechanism of AAI inhibition by local activation of S1P1 that is independent of the chain of events leading to efficient clonal T cell expansion.

Local i.t. delivery of S1P1 receptor agonist suppresses recruitment signals for activated T cell and eosinophil in the airways.

We tested whether or not the impairment of effector/memory T cell and eosinophil accumulation in lungs was due to the alteration of recruitment signals after local activation of S1P1. To do so, the concentrations of 'Thymus- and Activation-Regulated Chemokine' (TARC; CCL17) and 'Regulated upon Activation, Normal T-cell Expressed, and Secreted*' (*RANTES; CCL5) were determined in BALF twenty-four hours following the last i.t. delivery of OVA in OVA/OVA mice treated i.t. or i.p. with CYM-5442 or vehicle. While low in BALF of Saline/OVA mice (not shown), concentrations of TARC (Fig 5D) and RANTES (Fig 5E) were 15 ng/ml and 175 pg/ml, respectively, in OVA/OVA mice. Intra-tracheal delivery of CYM-5442 strongly suppressed the concentrations of TARC and RANTES by approximatively 75% in the BALF. As predicted by experiments with systemic agonist, i.p. delivery of CYM-5442 did not decrease TARC or RANTES levels compared to the i.p. vehicle treatment. Thus, secreted recruitment signals are significantly modulated by local i.t. delivery of an S1P1-specific agonist.

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Airway delivery of S1P prodrugs interferes with pulmonary immune response to alleviate immunopathology (Idzko et al., 2006; Marsolais et al., 2008), but attribution of function to specific S1P receptors has remained undefined. Using a specific S1P1 agonist with physical properties allowing airways delivery, we showed that S1P1 pharmacological activation in the airways contributes to alleviate AAI. Mechanistically, potent local anti-inflammatory activity of S1P1 agonist cannot be explained only by alterations of antigen presentation by DCs or by the induction of peripheral blood lymphopenia. Current data suggest that S1P1-induced inhibition of chemokine production and release in the airways may be an essential component of inflammation suppression by S1P prodrugs.

Specific S1P1 activation inhibits AAI. A number of in vitro studies suggest anti-inflammatory properties of S1P1 in macrophages (Hughes et al., 2008) and endothelial cells (Bolick et al., 2005). In vivo, S1P1, 3-5 agonist pro-drugs like FTY720 inhibit inflammation in models of experimental autoimmune encephalopathy (Papadopoulos et al., 2009), AAI (Idzko et al., 2006) and pulmonary viral infections (Marsolais et al., 2008; Marsolais et al., 2009), but contribution of single S1P receptors for alleviation of pulmonary immune response has remained elusive. Importantly, delivery of a non-phosphorylatable sphingosine analog does not inhibit T cell accumulation in lungs of mice infected with influenza virus, strongly supporting that S1P receptors are required for

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

immunomodulation in the airways (Marsolais et al., 2008). Moreover, S1P1 mRNA is expressed in the pulmonary tissue (Zhang et al., 1999). In accordance with literature we show, for the first time, that significant anti-inflammatory effects can be achieved with local i.t. delivery of a selective S1P1 receptor agonist.

Because local S1P1 activation in the airways quantitatively and qualitatively inhibits pulmonary inflammation similar to S1P prodrugs (Fig 2, (Marsolais et al., 2008), (Idzko et al., 2006)) we dissected the cellular events in AAI that could be shared by both S1P1-selective agonists and non-selective S1P pro-drugs (Fig 6). In contrast with AAL-R or FTY720 (Czeloth et al., 2005; Idzko et al., 2006; Marsolais et al., 2009), S1P1-specific chemical probes do not interfere with early steps of antigen presentation. While systemic delivery of an S1P1-specific agonist modestly alters DC trafficking in mice (Lan et al., 2005) and DC functions are modulated by the S1P3 receptor (Maeda et al., 2007; Niessen et al., 2008), no in vivo evidence supports a role for S1P1 located on DC to modulate their functions. Accordingly, local i.t. delivery of CYM-5442 during the early phase following influenza virus infection does not interfere with clonal expansion of T cells (Marsolais et al., 2008), and direct activation of S1P1 on DCs does not alter the movement of antigen-loaded DCs from lungs to lymph nodes (Fig 4). This is consistent with in vitro data showing no effect of the S1P1 agonist SEW2871 on DC migration and endocytosis (Maeda et al., 2007). Thus, our in vivo results show that S1P prodrugs-mediated alteration of antigen

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

presentation by DC is independent of S1P1 (Figs 3, 4 and 5) and that inhibition of AAI by S1P1 relies on modulation of other mechanisms.

S1P1 activation in the lungs inhibits the release of chemokines. S1P1 can alleviate the inflammatory response in vitro (Hughes et al., 2008) and in vivo (Lien et al., 2006). However, limited information is available regarding its mechanisms of action. The release of CCR4 ligands TARC and RANTES is decreased in the lungs by S1P1-specific agonist. Importantly, recruitment of Th-2 polarized $CD4^+$ T cells is mediated through ligands of CCR4 since this chemokine receptor is specifically expressed on Th-2 polarized T cells, as opposed to naïve, memory, Th-0 or Th-1 polarized T cells, that mainly express CXCR4 or CXCR3 (Sallusto et al., 1998). Of note, TARC induces mouse eosinophil chemotaxis, which would also explain the inhibition of eosinophil infiltration by local i.t. delivery of the S1P1 agonist (Borchers et al., 2002). A significant component of the immunosuppressive mechanism of local S1P1 activation is therefore through suppression of chemokine release within the pulmonary tissue.

Lymphocyte sequestration in secondary lymphoid organs partially explains the anti-inflammatory efficacy of pharmacological S1P1 activation. Indeed, systemic delivery of AAL-R (0.1 mg/kg) or the S1P1-specific agonists AUY954 (3mg/kg) or CYM-5442 (2mg/kg), which were all documented to induce significant lymphopenia at the doses used herein (Gonzalez-Cabrera et al., 2008;

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Marsolais et al., 2008; Pan et al., 2006), did not significantly inhibit eosinophil accumulation in lung during the recall phase of AAI (Figs 3 & 5), when compared with airway treatment (Figs 2 & 5). In the current study, compounds were administered 1h following allergen exposure, which allows early chemotactic factor release to occur. Our results contrast with ones others (Ble et al., 2009; Sawicka et al., 2003) who showed in similar AAI models that systemic delivery of FTY720 or S1P1-specific agonist inhibits T cell and eosinophil accumulation in the airways. This discrepancy is likely explained by the timing of treatment. Of note, Sawicka et al and Blé et al have administered mice with FTY720 prior to allergen exposure, thus sequestering T cells in non-draining lymph nodes, preventing their chemokine-driven recruitment in the airways. Moreover, FTY720 or AUY954-induced enhancement of barrier integrity prior to allergen exposure might also have inhibited inflammation in their systems (Ble et al., 2009). Thus, in our study, chemical probe delivery following allergen exposure revealed a critical role for S1P1 in the control of ongoing AAI by blunting chemokine release and recruitment of T cells and eosinophils.

Classically, systemic delivery of S1P pro-drugs or specific S1P1 agonists were shown to induce immunosuppression by sequestering lymphocytes in lymph nodes and Peyer's patches, preventing T and B cells from reaching the primary site of insult (Mandala et al., 2002; Sanna et al., 2004). On this basis, FTY720 was tested for efficacy in models of solid grafts, only to observe rejection with massive accumulation of myeloid leukocytes (Sis et al., 2008). This is similar

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

to what we observed with systemic delivery of S1P prodrugs or S1P1-specific agonists, with systemic doses sufficient to induce profound lymphopenia being impotent for inhibition of myeloid leukocyte infiltration. In more recent studies, airway delivery of S1P prodrugs has proven efficient at alleviating myeloid and lymphoid leukocyte accumulation in lungs of mice infected with an influenza virus (Marsolais et al., 2009; Marsolais and Rosen, 2009), or undergoing AAI (Idzko et al., 2006). The current experiments define specific molecular events in local S1P1 modulation of inflammatory cell recruitment. Systemic dosage of S1P1 agonists (Pan et al., 2006; Sanna et al., 2004) has been titrated for induction and maintenance of lymphopenia. However, accumulating evidence supports differential distribution of S1P receptor probes in various tissues (Gonzalez-Cabrera et al., 2008; Meno-Tetang et al., 2006), supporting potential distributional contributions to tissue-dependent modulatory activities.

In this study, we used a water-soluble tartaric salt of CYM-5442 to determine if local activation of S1P1 in the airways could contribute to inhibit AAI. Importantly, the half-life of CYM-5442 in the lungs after local i.t. delivery is the same as that measured by systemic delivery (Gonzalez-Cabrera et al., 2008). CYM-5442 does not preferentially accumulate in the lungs like other S1P receptor chemical probes (Meno-Tetang et al., 2006); and local effects can thus be dissociated from systemic effects using different routes of delivery. Moreover, in contrast to S1P, CYM-5442 induces significant S1P1 ubiquitination (Gonzalez-Cabrera et al., 2008), which is consistent with receptor internalization and

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

prolonged intracellular signaling (Mullershausen et al., 2009). Although in vivo selectivity is often hard to assess, CYM-5442 has high S1P1 selectivity upon S1P2-5, without antagonistic activities (at 10μM) on S1P1-5 (Gonzalez-Cabrera et al., 2008) and (The Scripps Research Institute Molecular Screening Center). In addition, in vivo selectivity is supported by the ability of CYM-5442 to induce lymphopenia (S1P1-specific effect), which can be competed using S1P1-specific antagonist W146 (Gonzalez-Cabrera et al., 2008). In view that CYM-5442-induced lymphopenia is competed in vivo with a selective antagonist in a dose-range similar to that used in the current study, we expect off target effects of the chemical probe to be minimal in the current setting.

Here, we show a new mechanism of inflammatory modulation by S1P prodrugs that involves S1P1 activation leading to suppression of cytokine production in the lung. Further characterization of S1P1 cellular distribution in the pulmonary environment and the molecular basis of modulation of allergic inflammation could ultimately lead to specific new therapies for pulmonary immunopathology.

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors would like to acknowledge the technical assistance of the personnel from TSRI Flow Cytometry Core Facility and of Serge Simard (Biostatistician; CRIUCPQ).

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Participated in research design: Marsolais, Yagi, Rosen

Conducted experiments: Marsolais, Yagi, Kago, Leaf

Performed data analysis: Marsolais, Yagi

Wrote or contributed to the writing of the manuscript: Marsolais, Yagi, Rosen

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

References

Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, Maceyka M, Jiang H, Luo C, Kordula T, Milstien S and Spiegel S Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature* **465**(7301):1084-1088.

Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S and Panettieri RA, Jr. (2001) Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *Faseb J* **15**(7):1212-1214.

Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N and Baumruker T (2003) Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *The Journal of biological chemistry* **278**(48):47408-47415.

Ble FX, Cannet C, Zurbruegg S, Gerard C, Frossard N, Beckmann N and Trifilieff A (2009) Activation of the lung S1P(1) receptor reduces allergen-induced plasma leakage in mice. *Br J Pharmacol*.

Bolick DT, Srinivasan S, Kim KW, Hatley ME, Clemens JJ, Whetzel A, Ferger N, Macdonald TL, Davis MD, Tsao PS, Lynch KR and Hedrick CC (2005) Sphingosine-1-phosphate prevents tumor necrosis factor-{alpha}-mediated monocyte adhesion to aortic endothelium in mice. *Arterioscler Thromb Vasc Biol* **25**(5):976-981.

Borchers MT, Ansay T, DeSalle R, Daugherty BL, Shen H, Metzger M, Lee NA and Lee JJ (2002) In vitro assessment of chemokine receptor-ligand interactions mediating mouse eosinophil migration. *J Leukoc Biol* **71**(6):1033-1041.

Brinkmann V, Cyster JG and Hla T (2004) FTY720: sphingosine 1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. *Am J Transplant* **4**(7):1019-1025.

Czeloth N, Bernhardt G, Hofmann F, Genth H and Forster R (2005) Sphingosine-1-phosphate mediates migration of mature dendritic cells. *J Immunol* **175**(5):2960-2967.

Gonzalez-Cabrera PJ, Jo E, Sanna MG, Brown S, Leaf N, Marsolais D, Schaeffer MT, Chapman J, Cameron M, Guerrero M, Roberts E and Rosen H (2008) Full pharmacological efficacy of a novel S1P1 agonist that does not require S1P-like headgroup interactions. *Mol Pharmacol* **74**(5):1308-1318.

Graler MH, Bernhardt G and Lipp M (1998) EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* **53**(2):164-169.

Hughes JE, Srinivasan S, Lynch KR, Proia RL, Ferdek P and Hedrick CC (2008) Sphingosine-1-phosphate induces an antiinflammatory phenotype in macrophages. *Circ Res* **102**(8):950-958.

Idzko M, Hammad H, van Nimwegen M, Kool M, Muller T, Soullie T, Willart MA, Hijdra D, Hoogsteden HC and Lambrecht BN (2006) Local application of

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* **116**(11):2935-2944.

Jolly PS, Bektas M, Olivera A, Gonzalez-Espinosa C, Proia RL, Rivera J, Milstien S and Spiegel S (2004) Transactivation of sphingosine-1-phosphate receptors by FcepsilonRI triggering is required for normal mast cell degranulation and chemotaxis. *J Exp Med* **199**(7):959-970.

Lan YY, De Creus A, Colvin BL, Abe M, Brinkmann V, Coates PT and Thomson AW (2005) The sphingosine-1-phosphate receptor agonist FTY720 modulates dendritic cell trafficking in vivo. *Am J Transplant* **5**(11):2649-2659.

Lien YH, Yong KC, Cho C, Igarashi S and Lai LW (2006) S1P(1)-selective agonist, SEW2871, ameliorates ischemic acute renal failure. *Kidney Int* **69**(9):1601-1608.

Liu G, Burns S, Huang G, Boyd K, Proia RL, Flavell RA and Chi H (2009) The receptor S1P1 overrides regulatory T cell-mediated immune suppression through Akt-mTOR. *Nat Immunol* **10**(7):769-777.

Liu H, Sugiura M, Nava VE, Edsall LC, Kono K, Poulton S, Milstien S, Kohama T and Spiegel S (2000) Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *The Journal of biological chemistry* **275**(26):19513-19520.

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N and Schuler G (1999) An advanced culture method for generating large quantities of

highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* **223**(1):77-92.

Maeda Y, Matsuyuki H, Shimano K, Kataoka H, Sugahara K and Chiba K (2007) Migration of CD4 T cells and dendritic cells toward sphingosine 1-phosphate (S1P) is mediated by different receptor subtypes: S1P regulates the functions of murine mature dendritic cells via S1P receptor type 3. *J Immunol* **178**(6):3437-3446.

Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, Thornton R, Shei GJ, Card D, Keohane C, Rosenbach M, Hale J, Lynch CL, Rupprecht K, Parsons W and Rosen H (2002) Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**(5566):346-349.

Marsolais D, Hahm B, Edelmann KH, Walsh KB, Guerrero M, Hatta Y, Kawaoka Y, Roberts E, Oldstone MB and Rosen H (2008) Local not systemic modulation of dendritic cell S1P receptors in lung blunts virus-specific immune responses to influenza. *Mol Pharmacol* **74**(3):896-903.

Marsolais D, Hahm B, Walsh KB, Edelmann KH, McGavern D, Hatta Y, Kawaoka Y, Rosen H and Oldstone MB (2009) A critical role for the sphingosine analog AAL-R in dampening the cytokine response during influenza virus infection. *Proc Natl Acad Sci U S A* **106**(5):1560-1565.

Marsolais D and Rosen H (2009) Chemical modulators of sphingosine-1-phosphate receptors as barrier-oriented therapeutic molecules. *Nature reviews*.

Massion PP, Linden A, Inoue H, Mathy M, Grattan KM and Nadel JA (1996) Dimethyl sulfoxide decreases interleukin-8-mediated neutrophil recruitment in the airways. *Am J Physiol* **271**(5 Pt 1):L838-843.

Meno-Tetang GM, Li H, Mis S, Pyszczynski N, Heining P, Lowe P and Jusko WJ (2006) Physiologically based pharmacokinetic modeling of FTY720 (2-amino-2[2-(-4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) in rats after oral and intravenous doses. *Drug Metab Dispos* **34**(9):1480-1487.

Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D and Seuwen K (2009) Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat Chem Biol* **5**(6):428-434.

Niessen F, Schaffner F, Furlan-Freguia C, Pawlinski R, Bhattacharjee G, Chun J, Derian CK, Andrade-Gordon P, Rosen H and Ruf W (2008) Dendritic cell PAR1-S1P3 signalling couples coagulation and inflammation. *Nature* **452**(7187):654-658.

Nishiuma T, Nishimura Y, Okada T, Kuramoto E, Kotani Y, Jahangeer S and Nakamura SI (2008) Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model. *Am J Physiol Lung Cell Mol Physiol*.

Pan S, Mi Y, Pally C, Beerli C, Chen A, Guerini D, Hinterding K, Nuesslein-Hildesheim B, Tuntland T, Lefebvre S, Liu Y, Gao W, Chu A, Brinkmann V, Bruns C, Streiff M, Cannet C, Cooke N and Gray N (2006) A monoselective sphingosine-1-phosphate receptor-1 agonist prevents

allograft rejection in a stringent rat heart transplantation model. *Chem Biol* **13**(11):1227-1234.

Papadopoulos D, Rundle J, Patel R, Marshall I, Stretton J, Eaton R, Richardson JC, Gonzalez MI, Philpott KL and Reynolds R (2009) FTY720 ameliorates MOG-induced experimental autoimmune encephalomyelitis by suppressing both cellular and humoral immune responses. *J Neurosci Res*.

Petrache I, Natarajan V, Zhen L, Medler TR, Richter AT, Cho C, Hubbard WC, Berdyshev EV and Tuder RM (2005) Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med* **11**(5):491-498.

Roviezzo F, Del Galdo F, Abbate G, Bucci M, D'Agostino B, Antunes E, De Dominicis G, Parente L, Rossi F, Cirino G and De Palma R (2004) Human eosinophil chemotaxis and selective in vivo recruitment by sphingosine 1-phosphate. *Proc Natl Acad Sci U S A* **101**(30):11170-11175.

Sallusto F, Lenig D, Mackay CR and Lanzavecchia A (1998) Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* **187**(6):875-883.

Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N and Rosen H (2004) Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *The Journal of biological chemistry* **279**(14):13839-13848.

Sanna MG, Wang SK, Gonzalez-Cabrera PJ, Don A, Marsolais D, Matheu MP, Wei SH, Parker I, Jo E, Cheng WC, Cahalan MD, Wong CH and Rosen H (2006) Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral S1P1 antagonist in vivo. *Nat Chem Biol* **2**(8):434-441.

Sawicka E, Zuany-Amorim C, Manlius C, Trifilieff A, Brinkmann V, Kemeny DM and Walker C (2003) Inhibition of Th1- and Th2-mediated airway inflammation by the sphingosine 1-phosphate receptor agonist FTY720. *J Immunol* **171**(11):6206-6214.

Sis B, Grynoch R, Murray AG, Campbell P and Solez K (2008) Antibody-mediated rejection with a striking interstitial monocyte/macrophage infiltration in a renal allograft under FTY720 treatment. *Am J Kidney Dis* **51**(1):127-130.

Skoura A, Sanchez T, Claffey K, Mandala SM, Proia RL and Hla T (2007) Essential role of sphingosine 1-phosphate receptor 2 in pathological angiogenesis of the mouse retina. *J Clin Invest* **117**(9):2506-2516.

Zhang G, Contos JJ, Weiner JA, Fukushima N and Chun J (1999) Comparative analysis of three murine G-protein coupled receptors activated by sphingosine-1-phosphate. *Gene* **227**(1):89-99.

Footnotes:

This work was supported in part by the National Institutes of Health [AI05509] [AI074564] [MH074404]; by a grant from Kyorin Pharmaceuticals [SFP-1799]; by *Le Fonds de la Recherche en Santé du Québec* (FRSQ); the *Respirarory Health Network of FRSQ* and by *La Chaire de Pneumologie de la Fondation J-D Bégin de l'Université Laval et de la Fondation de l'IUCPQ*.

David Marsolais and Saiko Yagi contributed equally to the manuscript.

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1: Experimental Model. Mice were sensitized on days 0 and 7 by i.p. injections of ovalbumin (OVA) coupled to the adjuvant aluminum hydroxide (Alum). Recall phase consisted in i.t. delivery of OVA, once daily, on consecutive days, starting on day 19. Mice sensitized and challenged with OVA (OVA/OVA mice) developed allergic airway inflammation. When required, sham procedure was performed and consisted in injecting mice with a mixture of saline and aluminum hydroxide prior to OVA i.t. challenges (Saline/OVA mice). Depending on the experiment, three to five i.t. OVA challenges were performed and mice were euthanized 24h following the last i.t. OVA delivery (on days 22 and 24, respectively) for analyses. AUY954 or AAL-R were administered once daily starting 1h post-OVA i.t. delivery. To account for the short in vivo half life of CYM-5442, this compound was delivered 1h and 13h after each i.t. delivery of OVA.

Figure 2: Local activation of S1P receptors efficiently inhibits eosinophilic airway inflammation. (**A-D**) C57BL/6J mice were either left intact (CTR), submitted to the sham procedure (Saline/OVA) or to the procedure inducing allergic airway inflammation (OVA/OVA). Saline/OVA or OVA/OVA mice were treated i.t. with 50µl of water (VEH) or AAL-R (0.1 $mg*kg^{-1}$) 1 hour following each daily i.t. delivery of OVA, during a 3 days recall response. Mice were euthanized 24 hours following the last i.t. delivery of OVA. (**A**) The accumulation of $CD4^+$ T cells and (**B**) eosinophils was inhibited in the BALF by the AAL-R treatment,

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

when compared to VEH, in the OVA/OVA mice. Also, (**C**) The number of $CD11c^+$ dendritic cells (DCs) and of (**D**) $CD4^+$ T cells were significantly decreased in mediastinal lymph nodes (MLN) by AAL-R treatment, when compared to VEH in OVA/OVA mice. n = 3-5 mice per group; bars represent means ± SEM; *Significantly different from VEH, $p < 0.05$.

Figure 3: Systemic delivery of AAL-R or AUY954 is impotent for control of allergic airway inflammation. Balb/c mice were left intact (CTR), or submitted to the procedure inducing allergic airway inflammation (OVA/OVA). OVA/OVA mice were administered by gavage with water (VEH), AAL-R (0.1 $mg*kg^{-1}$) or AUY954 (AUY; 3 $mg*kg^{-1}$) 3h prior to each daily i.t. instillation of OVA, during a 5 days recall response. Mice were euthanized 24 hours following the last i.t. delivery of OVA. Systemic delivery of AAL-R or AUY954 did not significantly inhibit of the accumulation of (**A**) $CD11c^+$ cells or (**B**) $CD4^+$ T cells in the draining mediastinal lymph nodes, when compared to VEH. AAL-R and AUY954 did not strongly reduce the accumulation of (**C**) T cells, or (**D**) eosinophils in the BALF. n = 4-6 mice per group; bars represent means ± SEM; *Significantly different from VEH, $p < 0.05$.

Figure 4: Neither local nor systemic S1P1-specific activation impair dendritic cell movement from lungs to mediastinal lymph nodes. (**A**) Mice were administered by gavage with AUY954 (3mg/kg), then Alexa Fluor 647-OVA (AF647) (50µg) was administered i.t.. Twenty-four hours later, mice were

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

euthanized and $CD11c^+$ $AF647^+$ cells were detected by flow cytometry in the mediastinal lymph nodes. (**B-C**) BMDCs were incubated for 18h in the presence of OVA (0.2 $mg*ml^{-1}$) in DC medium without FBS containing either 0.1% DMSO or AUY954 (1µM). BMDCs were then stained with CFSE and 50µl of a $1.5*10^6$ $cells*ml^{-1}$ suspension was administered i.t. to intact mice. Mice were euthanized 48h later. No CFSE signal could be detected in mediastinal lymph nodes (MLN) of in PBS-treated DCs (not shown). (**A**) Systemic AUY954 treatment did not affect movement of DCs from lungs to mediastinal lymph nodes. (**B**) The number of $CFSE^+$ $CD11c^+$ DCs in the mediastinal lymph nodes was not affected by incubation with AUY954, when compared to DMSO treatment, (**C**) nor was the total number of $CD4^+$ T cells. n = 3 mice per group; bars represent means ± SEM; *Significantly different, $p < 0.05$.

Figure 5: Local i.t. delivery of CYM-5442 inhibits the development of allergic airway inflammation and chemokine release in the airways. Balb/c mice were submitted to the sham procedure (Saline/OVA) or to the procedure inducing allergic airway inflammation (OVA/OVA). OVA/OVA mice were treated i.t. or i.p. with water (VEH) or CYM-5442 (2 $mg*kg^{-1}$) 1h and 13h following the daily i.t. delivery of OVA, during a 5 days recall response. Mice were euthanized 24 hours following the last i.t. delivery of OVA. The number of (**A**) total $CD4^+$ T cells, (**B**) eosinophils, (**C**) Effector/Memory $CD44^+$ $CD4^+$ T cells were quantified in BALF. Intra-tracheal delivery of CYM5442 is efficient at inhibiting $CD44^+$ $CD4^+$ T cells and eosinophils in BALF when compared to i.p. delivery. (**D**) TARC and (**E**)

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

RANTES levels were also quantified in BALF. RANTES was not consistently detected in Saline/OVA mice (< 12pg/ml, when detected), while basal levels of TARC were 148±55 pg/ml (not shown). When compared to VEH treatments, i.t. but not i.p. delivery of CYM5442 strongly inhibited the release of these 2 cytokines in the BALF. n = 3-5 mice per group, experiment was repeated twice; bars represent means ± SEM; *Significantly different from VEH, $p < 0.05$.

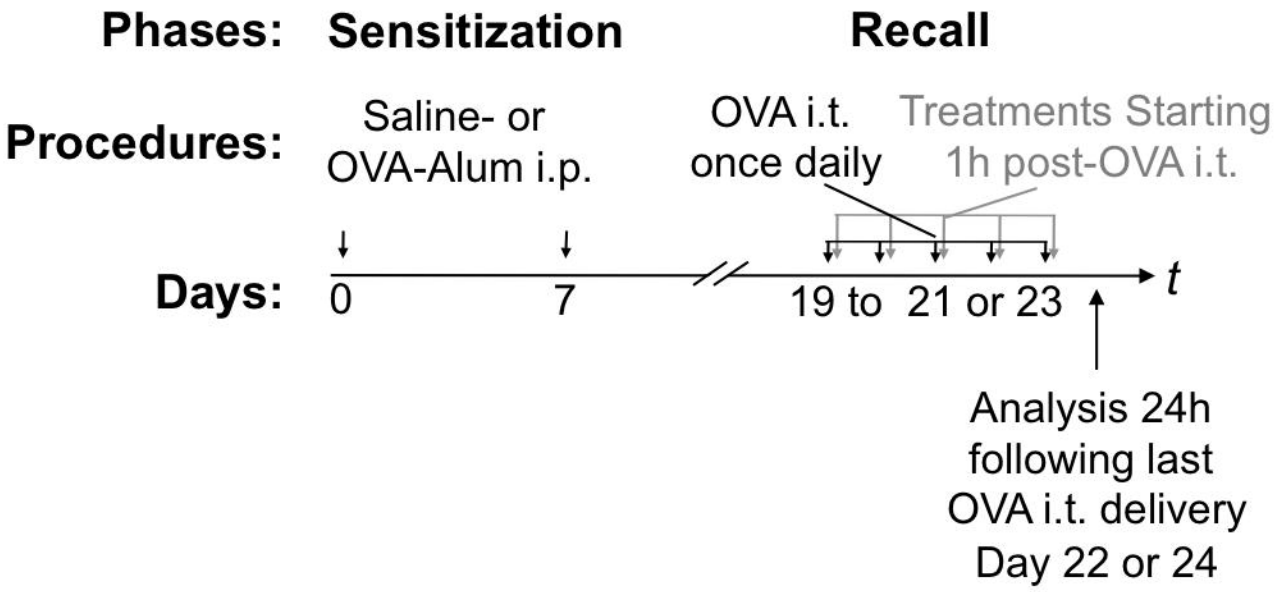
Figure 6: Chemical modulation of S1P1 interrupts AAI by inhibiting the release of chemokines in the airways. In sensitized mice, airway allergen exposure triggers rapid release of chemokines and proinflammatory cytokines by several cell subtypes in the airways including mast cells, macrophages, dendritic cells, epithelial and endothelial cells. Antigen is uptaken by dendritic cells that migrate towards draining lymph nodes where they present antigen to lymphocytes. There, lymphocytes undergo rapid expansion, egress from lymph nodes to efferent lymphatic vessels and then reach blood circulation. Because chemokines are released in the lung, activated T cells and eosinophils accumulate in the airways where they synergize with cells already in that location to further recruit leukocytes. Eosinophils, will release a plethora of factors inducing tissue remodeling thus contributing to the development of airway hyperresponsiveness. Current and documented experiments (Idzko et al., 2006; Marsolais et al., 2008; Marsolais et al., 2009) support that i.t. delivery of non-selective S1P prodrugs interfere with antigen presentation process to inhibit pulmonary immune response. Here we show that selective S1P1 activation in the

Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

airways interferes with the development of AAI by inhibiting the release of chemokines in the airways, while not affecting steps of antigen presentation.

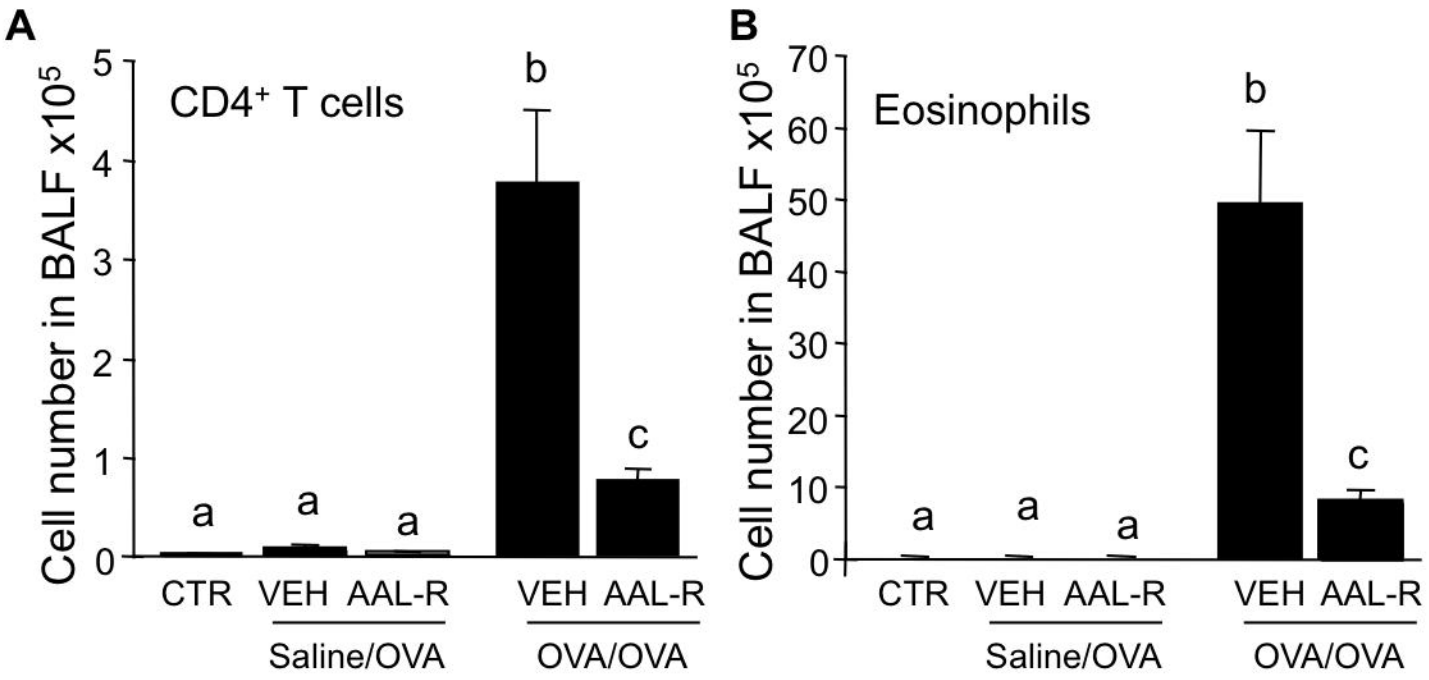
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



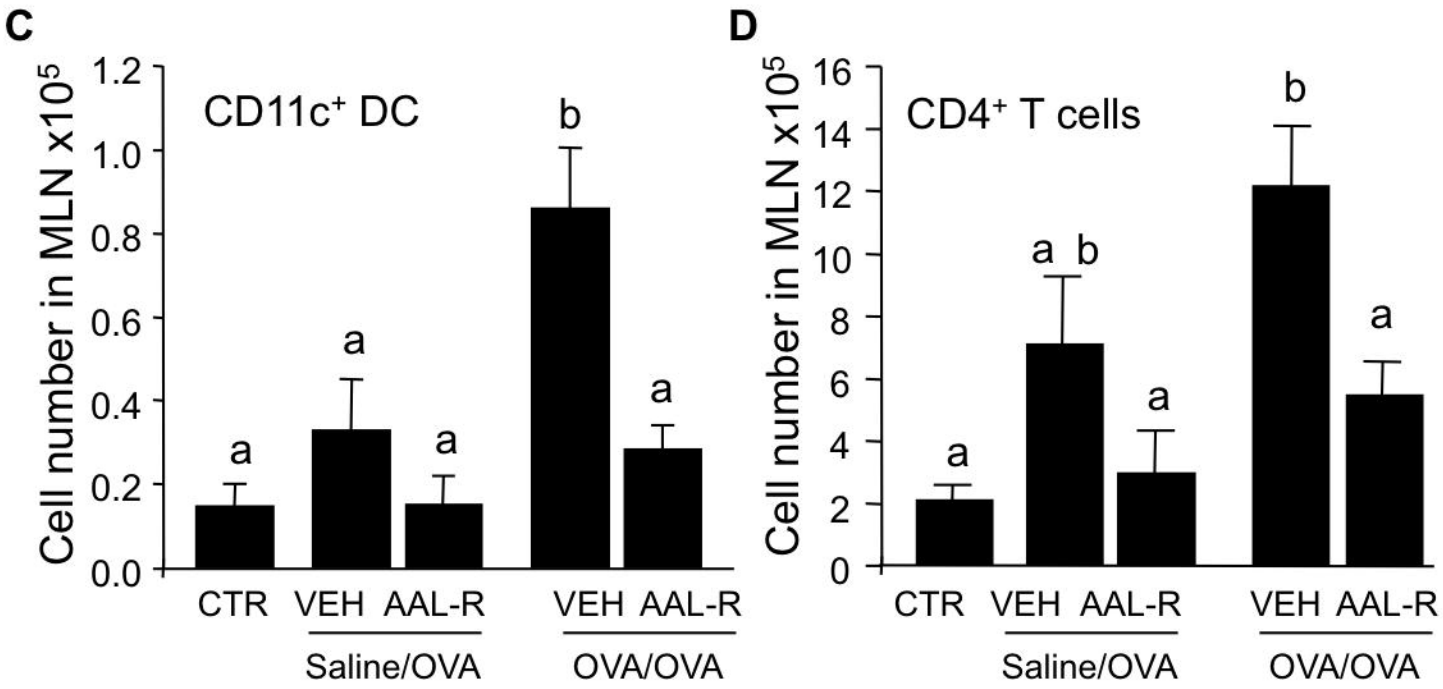
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2 part 1



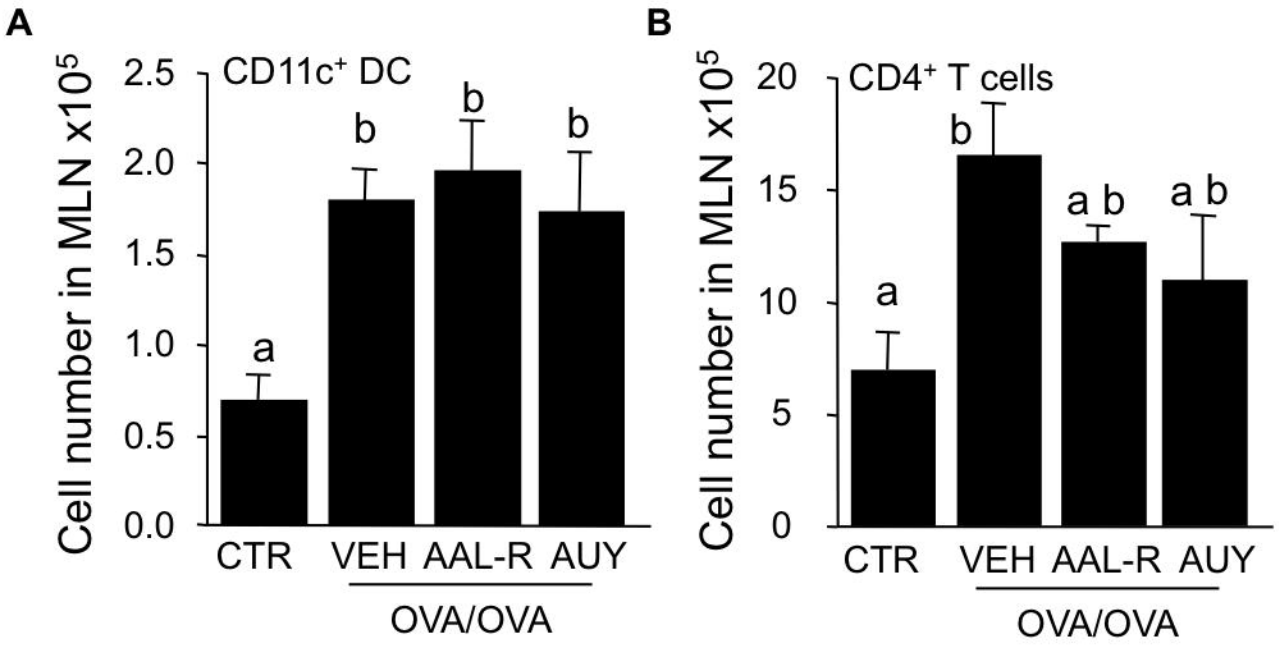
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2 part 2



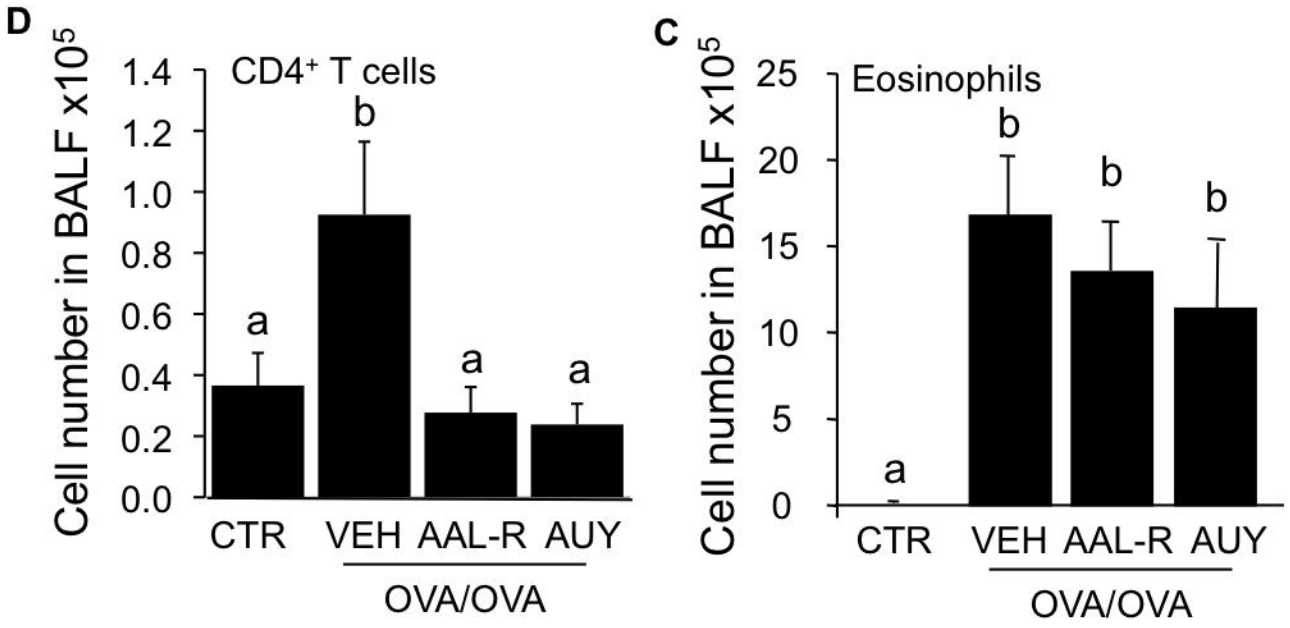
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3 part 1



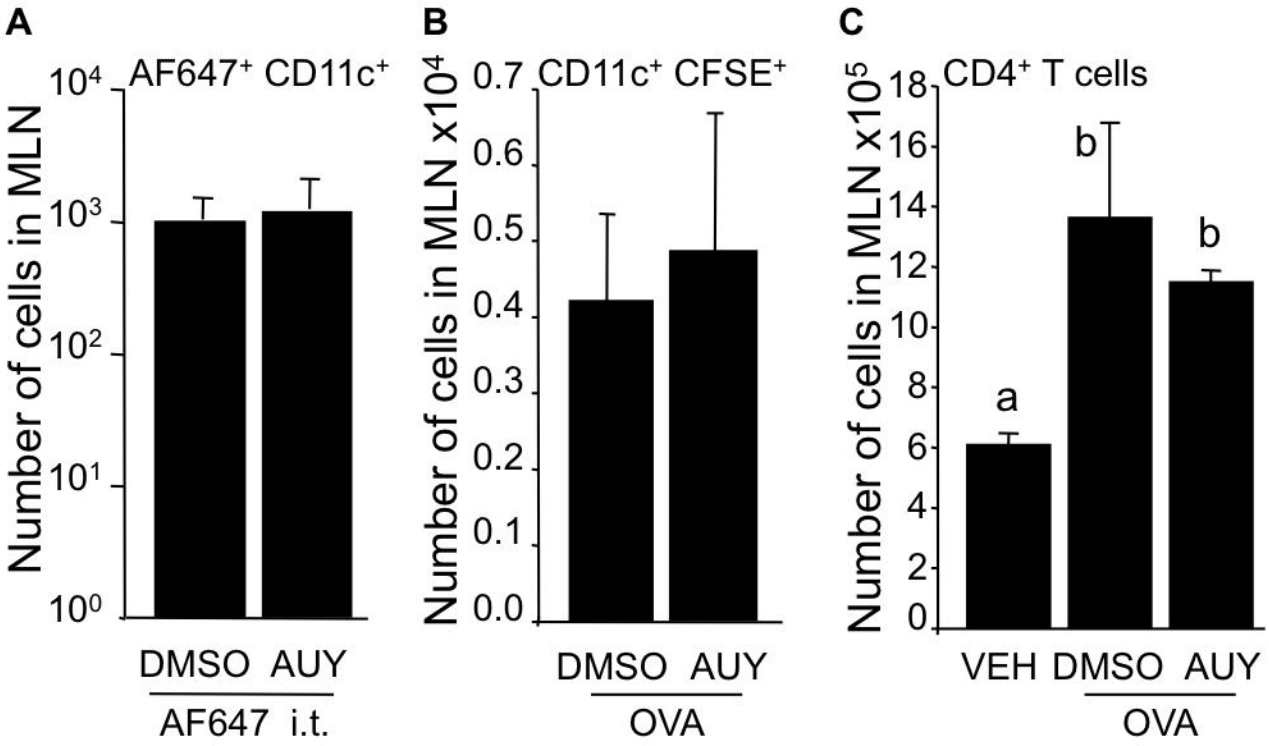
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3 part 2



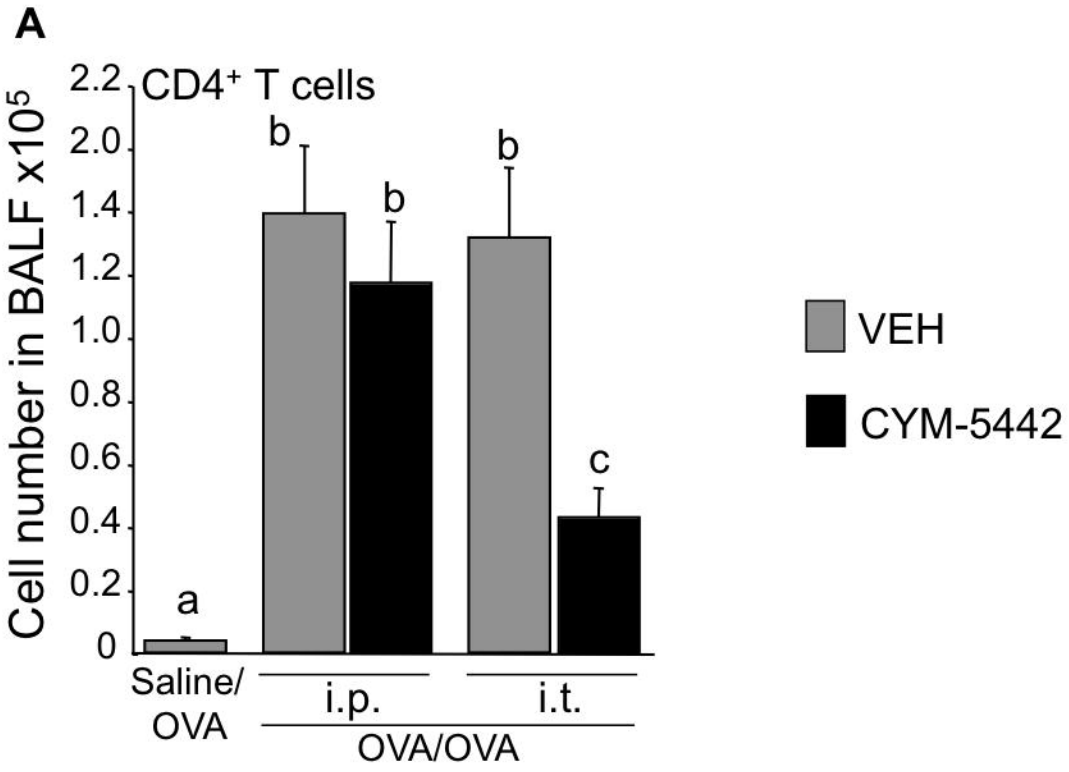
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



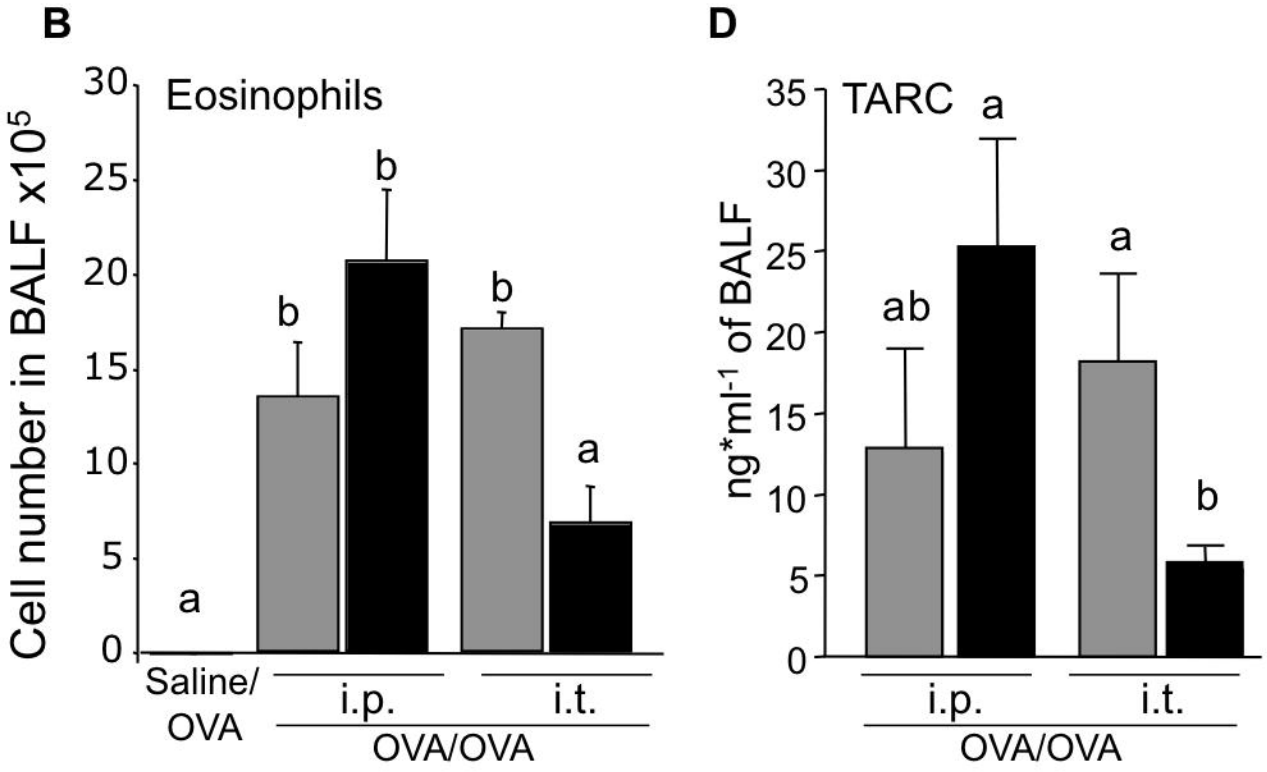
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5 part 1



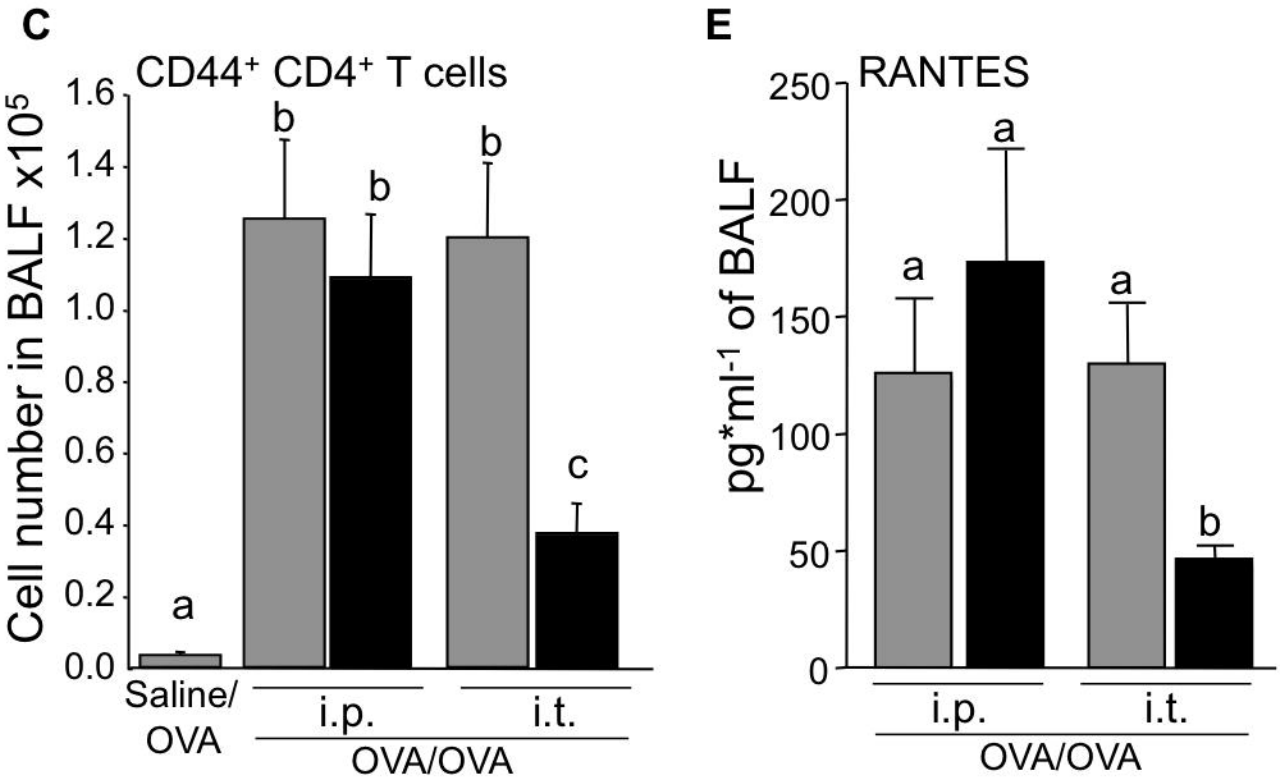
Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5 part 2



Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5 part 3



Molecular Pharmacology Fast Forward. Published on October 8, 2010 as DOI: 10.1124/mol.110.066811
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6