Molecular Pharmacology Fast Forward. Published on June 24, 2008 as doi:10.1124/mol.108.048769
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MOL#48769

Local not systemic modulation of dendritic cell S1P receptors in lung blunts virus-specific immune responses to influenza

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MOL#48769

Running Title: T cell suppression by S1P analog modulation of DCs

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Text pages: 20 (Introduction to end of discussion)
Figures: 7
Supplementary Files: 1
References: 40
Words in abstract: 210
Words in introduction: 530
Words in discussion: 1256

Abbreviations: DC, dendritic cell; S1P, Sphingosine-1-phosphate; FITC, Fluorescein isothiocyanate; CTL, cytotoxic T lymphocyte; TNF, tumor necrosis factor; LCMV, lymphocytic choriomeningitis virus; NA, neuraminidase; tg, transgenic; GFP, green fluorescent protein; AAL, 2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol; AFD, 2-amino-4-(4-(heptyloxy)phenyl)-2-methylbutyl dihydrogen phosphate; IFN, interferon; MLN, mediastinal lymph node; MHC, Major Histocompatibility Complex; BM, bone marrow.

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Abstract

The mechanism by which locally delivered sphingosine analogs regulate host response to localized viral infection has never been addressed. In this report, we show that intra-tracheal (i.t.) delivery of chiral sphingosine analog AAL-R or its phosphate ester inhibits the T cell response to influenza-virus infection. In contrast, neither intra-peritoneal (i.p.) delivery of AAL-R nor i.t. instillation of the non-phosphorylable stereoisomer AAL-S suppressed virus-specific T cell response, indicating that in vivo phosphorylation of AAL-R and S1P receptor modulation in lungs are essential for immunomodulation. I.t. delivery of water soluble $S1P_1$ receptor agonist at doses sufficient to induce systemic lymphopenia did not inhibit virus-specific T cell response indicating that $S1P_1$ is not involved in the immunosuppressive activities of AAL-R and that immunosuppression acts independently of naïve lymphocyte recirculation. Accumulation of dendritic cells (DCs) in draining lymph nodes was inhibited by i.t. but not i.p. delivery of AAL-R. Direct modulation of DCs is demonstrated by the impaired ability of virus-infected bone-marrow derived DCs treated in vitro with AAL-R to trigger in vivo T cell response after adoptive transfer to the airways. Thus, our results suggest that locally delivered sphingosine analogs induce immunosuppression by modulating S1P receptors other than $S1P_1$ or $S1P_2$ on dendritic cells in the lungs after influenza virus infection.

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INTRODUCTION

Sphingosine, sphingosine 1-phosphate (S1P) and their synthetic analogs emerged as major immunomodulatory molecules (Rosen and Goetzl, 2005), with therapeutic potential being tested in multiple sclerosis (Massberg and von Andrian, 2006) and allogenic transplantation (Budde et al., 2006). Ubiquitous expression of S1P receptors and pleiotropic effects on multiple physiological systems suggest that there are numerous mechanisms of systemic immunomodulation by this receptor-ligand system including a role for $S1P_1$ in T and B cell development and egress (Matloubian et al., 2004) and $S1P_1$-dependant enhancement of the endothelial barrier of sinus-lining endothelium (Mandala et al., 2002; Sanna et al., 2006; Wei et al., 2005). For instance, systemically and chronically delivered sphingosine analogs modulate S1P receptors (Sanna et al., 2004) after in vivo phosphorylation (Billich et al., 2003), leading to sequestration of naïve lymphocytes in secondary lymphoid organs (Wei et al., 2005), therefore introducing significant inefficiencies in the immune response to localized antigen.

An increasing body of evidence also suggests that modulation the S1P immunoregulatory axis at the primary site of an insult might have unique and potent immunosuppressive mechanisms. For instance, S1P, S1P prodrugs and specific S1P receptor agonists impair chemokine release and cell surface expression of adhesion molecules on endothelial cells at primary inflammatory sites (Awad et al., 2006; Bolick et al., 2005; Ledgerwood et al., 2008; Okazaki et

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al., 2002; Peng and RM, 2004; Yopp et al., 2005), and can interfere with the function of sentinel cells such as dendritic cells (DCs), macrophages, endothelial and epithelial cells.

The mechanisms employed by sphingosine analogs to influence cellular responses are numerous. Indeed, FTY720 was shown to modulate the activity of various enzymes such as sphingosine kinase 1 (Lee et al., 2004) and S1P Lyase (Bandhuvula et al., 2005), with potential for perturbing synthesis and degradation cascades of various sphingolipids. Synthetic sphingosine analog (R)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL-R) was shown be phosphorylated in vivo to (*R*)-2-amino-4-(4-(heptyloxy)phenyl)-2-methylbutyl dihydrogen phosphate (AFD-R) by sphingosine kinase 2 to induce cell death by acting on yet to be defined intracellular targets (Don et al., 2007), or be secreted after phosphorylation to act on G protein-coupled receptors $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$. Each of these S1P receptors has a specific anatomical and cellular distribution and couples to different signaling cascades during homeostasis, but little information is currently available on their respective contribution during pathologic conditions.

In view of the evidence that locally delivered sphingosine analogs can act through multiple pathways to affect biological processes, we sought to combine the use chemical probes and a novel system that allows monitoring influenza-virus specific T cell responses in vivo to the determine the molecular and cellular

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immunosuppressive mechanisms of locally delivered sphingosine analogs. We noted that the sphingosine analog AAL-R is rapidly phosphorylated to AFD-R in the lungs after intra-tracheal delivery, and acts through S1P receptors to impair DC function and proinflammatory cytokines release in the lungs. Inhibition of virus-specific T cell expansion acted independently of $S1P_1$ and $S1P_2$ receptor modulation as well as independently of naïve lymphocyte recirculation. S1P receptor-mediated impairment of DC activation led to reduction of DC accumulation in draining lymph nodes and ultimately to blunting of influenza virus-specific T cell amplification that might be of therapeutic interest.

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Material and Methods

Mice

Mice were bred and maintained in a closed breeding facility at The Scripps Research Institute. C57BL/6, C57BL/6 Thy1.1^{+}D^{b}GP$_{33–41}$ TCR-tg and C57BL/6 GFP^{+}D^{b}GP$_{33–41}$ TCR-tg were used in this study. The handling of all mice conformed to the requirements of the National Institutes of Health and The Scripps Research Institute animal research committee.

Compounds

AAL-R, AAL-S and AFD-R were synthesized according to published methods (Kiuchi et al., 2000; Kiuchi et al., 1998). Synthesis of CYM-5442 ((2-(4-(5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1H-inden-1-ylamino)ethanol) is described in details elsewhere (Gonzalez-Cabrera et al; Mol Pharmacol, submitted).

Virus

A mutant A/WSN/33 virus bearing the LCMV immunodominant T cell-specific GP33 and GP65 tandem sequence AAGGCTGTCTACAATTTTGCCACCTGTGGGGGACGCACAAUGGGTCTTAAG GGACCCGACATTTACAAAGGAGTTTACCAATTTAAGTCAGTGGAGTTTGAT between nucleotides 145 and 146 of WSN NA gene (termed FLU-LCMV) was generated by using plasmid-based reverse genetics (Neumann et al., 1999). The mutated region was sequenced to confirm the presence of the LCMV epitope

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sequences. Virus was amplified and titrated in Madin-Darby Canine Kidney (MDCK) cells and stored at -80ºC.

Cell transfer, virus infection, and administration of compounds

Male C57BL/6 mice, (7-9 weeks old) were adoptively transferred with $5x10^4$ virus-specific $CD8^+$ T cells (GFP^+ or $Thy1.1^+$ $GP33/CD8^+$ T cells) by injection in the tail vein. On the following day, mice were administered i.t. with $1x10^5$ PFU of FLU-LCMV under Isoflurane anesthesia. One hour post infection, mice were anesthetized with an injection of 0.5 mg/g 2-2-2 Tribromoethanol (Sigma) intraperitoneally (i.p.) for the i.t. delivery of compounds i.e. AAL-R, AAL-S, CYM-5442 or water as VEH; AFD-R or 2% beta-cyclodextrin in water as VEH. For some experiments, mice were administered i.p. with compounds or with the previously indicated VEH.

Isolation of cells from organs and $CD8^+$ T cells enrichment

Lungs or mediastinal lymph nodes (MLNs) were harvested from PBS-perfused mice. Organs were disrupted mechanically through a 100µm filter, and then red blood cells and lung tissue debris were removed with 0.83% ammonium chloride and 31.5% Percoll™ (GE Healthcare Life Sciences) treatments, respectively. For DC analysis, whole lungs or MLNs were digested with collagenase D (1 mg/ml, type II, Sigma-Aldrich) for 15 min at 37°C and then treated with 0.01 M of EDTA for 5 min before mechanical disruption. Spleens from either C57BL/6 $Thy1.1^+D^bGP_{33–41}$ TCR-tg or C57BL/6 $GFP^+D^bGP_{33–41}$ TCR-tg mice were

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processed to a single cell suspension as above. T cells were purified by negative selection using StemSep™ Mouse $CD8^+$ T Cell Enrichment Kit (StemCell Technologies Inc), (purity > 95 %).

AAL-R, AFD-R and cytokines quantification

For AFD-R and AAL-R quantification, lungs were homogenized in ice-cold methanol. Supernatant was prepared for liquid chromatography and mass spectrometry as previously described (Don et al., 2007). Lungs from ambulatory control mice spiked in with increasing amounts of AAL-R or AFD-R were processed for extraction identically and simultaneously to samples as quantification standards. For multiplexed ELISA analysis of cytokines, whole lungs were homogenized in PBS and processed for analysis by Quansys Biosciences.

Flow cytometric analysis

Detection of surface molecules was performed as previously described (Hahm et al., 2004). Surface marker antibodies were specific for murine CD8, CD11b, CD11c, CD25, CD90.1 (Thy1.1), CD45R (B220) (BD PharMingen and eBioscience). GP33 peptide stimulation of T cells and intracellular staining of TNF-α and IFN-γ in $CD8^+$ T cells were performed as previously described (Christen et al., 2004).

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DC transfer assay

DCs were derived from bone marrow (BM) cells by incubation for 10 days with rmGM-CSF as described (Lutz et al., 1999). Cultured DCs were incubated with 3 multiplicity of infection (MOI) of FLU-LCMV or PBS for 1h, washed and incubated for 2 h with VEH or AAL-R (500 nM). After being washed, 1 x 10^5 DCs were transferred i.t. into C57BL/6 mice that had received 5 x 10^4 Thy 1.1^+ GP33/$CD8^+$ T cells i.v. on the previous day. Mice were euthanized 6 days post instillation for analysis of virus-specific T cells content in lungs.

Statistical analysis

Unless otherwise stated, bars represent means ± SEM and averages were compared using a bidirectional unpaired student's t test with a 5% significance level. Star (*) was used to mark significant differences between 2 groups unless otherwise stated. n≥3 mice per group for all statistical analyses.

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RESULTS

Pulmonary targets mediate sphingosine analog-induced inhibition of T cell response

We developed a highly quantitative model to study the T cell responses to influenza-virus infection using an engineered influenza virus (A/WSN/33, H1N1) bearing CD4 and CD8 immunodominant epitopes of the lymphocytic choriomeningitis virus (LCMV) in its neuraminidase (NA) stalk (Hahm et al., 2007; Neumann et al., 1999) that was termed FLU-LCMV. The availability of T cell receptor transgenic mice for these 2 epitopes allows for monitoring and quantification of virus-specific T cell response in vivo as previously described elsewhere (Hahm et al., 2007). Using this in vivo model system, we investigated the mechanism of action of locally delivered sphingosine analog during pulmonary viral infection.

While few or no adoptively transferred FLU-LCMV specific $CD8^+$ T cells (GP33/$CD8^+$ T cells) can be detected in the lungs of non-infected mice, FLU-LCMV infection leads to accumulation of 2 to 5 x 10^4 FLU-LCMV specific $CD8^+$ T cells in the lungs 6 days post infection. Intra-tracheal instillation of chiral sphingosine analog AAL-R 1 hour following infection with recombinant FLU-LCMV virus reduced the percentage of virus-specific $CD8^+$ T cells in the lungs in a dose-dependant manner, with maximal inhibition occurring at approximately 0.3 mg/kg (Figure 1A). Administering a single 0.1 mg/kg dose of AAL-R i.t. profoundly inhibited pulmonary accumulation of total virus-specific $CD8^+$ T cells

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(Figure 1B) while i.p. delivery of the same (Figure 1B) or three-fold higher amount of AAL-R (See supplemental data) had no significant effect on virus-specific $CD8^+$ T cell accumulation in the lungs. Importantly, the kinetics of blood lymphopenia induced by local or systemic delivery of AAL-R did not differ significantly (Figure 1C). These results indicate that modulation of pulmonary, rather than systemic targets are required for T cell suppression and that inhibition of naïve T cell recirculation is not the mechanism by which AAL-R alters the influenza virus-induced pulmonary immune response.

Local phosphorylation of sphingosine analog and S1P receptor modulation is required for immunosuppression

Sphingosine analog AAL-R can be phosphorylated by sphingosine kinase 2 on its primary hydroxyl group, leading to generation of a broad $S1P_{1,\ 3\text{-}5}$ receptor agonist AFD-R. On the other hand, chiral *S* enantiomer of AAL-R, termed AAL-S, is not phosphorylated in vivo, and can be used as a tool to discriminate phosphorylation-dependent from phosphorylation-independent activities of sphingosine analogs (Brinkmann et al., 2002; Mandala et al., 2002). Mock or FLU-LCMV infected mice were administered with VEH, AAL-S or AAL-R. Mock infection resulted in no pulmonary accumulation of virus-specific $CD8^+$ T cells. On the other hand, 2.5 $x10^4$ virus-specific $CD8^+$ T cells could be detected in the lungs of mice 6 days after i.t. delivery of FLU-LCMV infection. While i.t. delivery of 2 mg/kg of AAL-R totally suppressed virus-specific T cell accumulation in the

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lungs, administration of a same dose of AAL-S had no significant effect when compared to vehicle (VEH) treatment (Figures 2A and 2B).

AAL-S inactivity was confirmed by assessment of cytokine release 2 days post infection with FLU-LCMV virus. When compared to ambulatory control mice, interleukin (IL)-6 (~600 fold) (Figure 3A) and monocyte chemoattractant protein (MCP)-1 (~300 fold) (Figure 3B) were prominently elevated by FLU-LCMV infection (Figure 3A). Both IL-6 and MCP-1 were strongly suppressed by AAL-R (52% and 59% inhibition, respectively) (Figure 3A-B). AAL-R also substantially reduced pulmonary content of IL-1β (47%) (Figure 3C) and TNF-α (41%) (Figure 3D), when compared to infected mice treated with VEH. In sharp contrast, instillation of AAL-S isomeric control did not reduce the extent of cytokine release triggered by FLU-LCMV infection supporting the requirement of sphingosine analogs' phosphorylation for immunomodulation to occur.

To provide direct evidence that AAL-R was phosphorylated in our system, we measured the amount of AFD-R produced in the lungs after i.t. delivery of AAL-R, by means of liquid chromatography-mass spectrometry (Figure 4). After i.t. delivery, AAL-R was rapidly phosphorylated to AFD-R in the lungs and remained at a molecular ratio of 6:1 (AFD-R:AAL-R) for at least 24 hours post-instillation, which agrees with published serum pharmacokinetics (Brinkmann et al., 2002; Mandala et al., 2002; Rosen et al., 2003). Intratracheal instillation of AAL-R resulted in a 10-fold higher AFD-R accumulation within the lungs at 30 min post-

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administration, when compared to i.p. delivery. Pulmonary AFD-R content was diminished by 80% after 24 hours, highlighting the transient nature of the treatment (Figure 4). The decreased compound concentration in the lungs likely reflects redistribution to the bloodstream and the interstitial fluids, which is in keeping with the systemic lymphopenia observed (Figure 1C). Considering the membrane impermeant character of AFD-R (Brinkmann et al., 2002), early modulation of pulmonary S1P receptors is the most likely immunosuppressive mechanism of locally delivered sphingosine analog.

To determine whether or not suppression of T cell accumulation in lungs was caused by direct modulation of S1P receptors, molar equivalent doses of AAL-R (0.1 mg/kg) or AFD-R (0.13 mg/kg) were administered i.t. following infection with FLU-LCMV. AFD-R displayed an equal potency to AAL-R for inhibiting pulmonary accumulation of virus-specific, IFN-γ and TNF-α-producing $CD8^+$ T lymphocytes (Figures 5A and 5B), indicating that immunosuppression did not require intracellular activities of either AAL-R or AFD-R (Don et al., 2007). Notably, virus-specific $CD8^+$ T cells were barely detectable in the lungs of mice treated i.t. with 0.26 mg/kg of AFD-R. Therefore, the mechanism by which sphingosine analog operates to control the pulmonary immune response proceeds with rapid local phosphorylation and local modulation of S1P receptors.

$S1P_1$ receptor activation does not inhibit T cell response to FLU-LCMV

To determine the involvement of $S1P_1$ in the modulation of virus-specific $CD8^+$ T cell response following infection, we used a recently developed water-soluble

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$S1P_1$ specific receptor agonist. CYM-5442 induces systemic lymphopenia (Figure 6A) and sequestration of T lymphocytes in non-draining lymph nodes 8 hours after i.t. delivery, showing in vivo effectiveness (Figure 6B). In accordance with documented lymphopenic response induced by $S1P_1$ specific agonists (Pan et al., 2006; Sanna et al., 2004), a single i.p. injection of CYM-5442 induced lymphopenia for least 18 hours (data not shown). In spite of systemic effects on naïve lymphocyte recirculation, i.t. delivery of CYM-5442 for up to 2 mg/kg i.t. (Figure 6C; data not shown) did not impair the percentage or total number of virus-specific $CD8^+$ T cells in the lungs 6 days post FLU-LCMV infection.

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Direct activity of AAL-R upon dendritic cells inhibits the immune response to FLU-LCMV

Upon pulmonary infection, DCs migrate from the lungs to the MLNs where they induce T cell expansion (Baumgarth and Kelso, 1996; Belz et al., 2004). Locally delivered AAL-R significantly reduced the number of virus-specific $CD8^+$ T cells in draining MLNs following infection with FLU-LCMV, indicating that AAL-R inhibited clonal expansion of T cells upon infection (data not shown). Intra-tracheal delivery of AAL-R did not reduce the numbers and viability DCs in the lungs (data not shown), but inhibited their accumulation in the MLNs (Figure 7A). Interestingly, i.p. delivery of AAL-R did not affect accumulation of DCs in MLNs (Figure 7A), further supporting that local modulation of S1P receptors in the lungs, but not systemic modulation, is responsible for T cell suppression.

To investigate whether AAL-R could directly act on DCs to influence host immune response to influenza virus, BMDCs were infected with FLU-LCMV and then treated with AAL-R in vitro. Virus-infected DCs instilled into the airways triggered accumulation of virus-specific $CD8^+$ T cells in the lungs 6 days post infection, whereas uninfected DCs did not (Figure 7B). Concomitant treatment of FLU-LCMV-infected DCs with AAL-R in vitro significantly suppressed the percentage of virus-specific $CD8^+$ T cells amplified in vivo by more than 70% (Figure 7B). BMDCs have the ability to phosphorylate AAL-R in vitro as we determined that 83% ± 0.9% of the species recovered from pooled cells and culture supernatant 4h after addition of AAL-R to BMDCs were the phosphate

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ester, which further supports that sphingosine analog could directly modulate DC activities of T cell stimulation upon influenza virus infection by acting on S1P receptors.

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DISCUSSION

In this report, we used chemical probes to delineate the in vivo molecular mechanism employed by locally delivered sphingosine analog to alter the immune response to viral infection. Using an LCMV epitope-tagged in vivo influenza model system, we determined that T cell suppression induced by locally delivered sphingosine analog has a mechanism independent of $S1P_1$ and $S1P_2$ activation and of naïve lymphocyte sequestration in the secondary lymphoid organs, but involves intra-pulmonary accumulation of S1P receptor modulatory molecule and modulation of DC activity.

The immunosuppressive mechanism of sphingosine analogs has been extensively studied in models of allograft rejection and autoimmunity and has focused on mechanisms of lymphocyte recirculation. However, very few data is currently available regarding the modulatory potential and mechanisms of sphingosine analogs in response to pathogenic viral infections. It was previously shown that the sphingosine analog FTY720 can induce redistribution of effector lymphocyte populations in lymph nodes during a systemic LCMV infection without interfering with clonal expansion, which is in accordance with results obtained in models of allograft rejection (Brinkmann et al., 2001). Here, the failure of systemic AAL-R dosing and specific $S1P_1$ receptor activation to induce immunosuppression shows that lymphopenia alone could not explain immunomodulation observed in the influenza virus infection model. Instead, our results support that targeting S1P receptors locally in lungs and on DCs is

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sufficient to inhibit proinflammatory cytokine release and virus-specific T cell responses triggered by Influenza virus infection. Direct evidence of DC function alterations by local modulation of the S1P immunoregulatory axis is provided by the inability of i.p. delivery of AAL-R to induce inhibition of DC accumulation in the draining lymph nodes, and by the ability of AAL-R to inhibit the immune response triggered by adoptively transferred infected DCs. Therefore, rather than non-specifically inducing sequestration of normally expanding lymphocytes in secondary lymphoid organs as previously shown during LCMV infection (Pinschewer et al., 2000), we observed that local delivery of a sphingosine analog directly at the primary site of infection impairs with the chain of events leading to clonal expansion of T cells in draining lymph nodes following viral infection.

This study is the first to provide mechanistic insights regarding the molecular mechanism of airway-delivered sphingosine analogs. The use of chemical probes has proven utility to determine the mechanisms and targets of sphingosine analogs. For instance, delivery of FTY720-phosphate was shown to induce lymphopenia by acting on S1P receptors (Mandala et al., 2002) and FTY720, AAL-R but not AAL-S could inhibit vascular-endothelial growth factor-induced leakage in vivo (Brinkmann et al., 2002). Moreover AAL-R but, not AAL-S nor AFD-R, could induce apoptosis of selected jurkat cells in vitro (Don et al., 2007). Indeed, while both AAL-R and AAL-S but not AFD-R penetrate the cells, only AAL-R can be phosphorylated intracellularly and therefore induce cell death.

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Translating these findings to our in vivo model system, we determined that local delivery of a sphingosine analog AAL-R in the airways provided sustained and compartmentalized source of the bioactive sphingosine-1-phosphate analog AFD-R, which can bind 4 of the five high affinity S1P receptors. Sphingosine kinase 1 is the major sphingosine isoform expressed in the lungs (Liu et al., 2000), and has a lower rate of phosphorylation for synthetic sphingosine analogs such as FTY720 and AAL-R, when compared to sphingosine kinase 2 (Billich et al., 2003; Zemann et al., 2006). Despite of this, we determined that sphingosine kinase activities in the lungs were sufficient to rapidly phosphorylate AAL-R and maintain pulmonary levels above the limit of quantification for at least 24 hrs (Figure 4). Intratracheal delivery of cell membrane impermeant S1P receptor modulator AFD-R by itself inhibited T cell responses after influenza virus infection, which strongly argues that receptor engagement, not intracellular drug effects, is involved in the modulation of DC function during viral pathogenesis. This conclusion is further supported by the observation that the cell permeant but non-phosphorylable sphingosine analog AAL-S did not induce suppression of T cell response.

Resolving the exact involvement of S1P receptor ligands in the regulation of pulmonary immune response in vivo is complexified by differential distribution of the 5 S1P receptors. Our results involving the S1P receptors in the modulation of DC function are supported by a number of in vitro and in vivo data. Indeed, $S1P_1$ and $S1P_3$ receptors are upregulated on DCs upon maturation (Czeloth et al.,

2005), and treatment with nanomolar concentrations of phosphorylated sphingosine analog FTY720-P, interferes with DC migration towards S1P, as well as with S1P-induced phagocytosis in vitro (Maeda et al., 2007). Most in vivo studies were however performed with non-phosphorylated FTY720, which can act independently of cell-surface S1P receptor modulation (Bandhuvula et al., 2005; Don et al., 2007; Dudek et al., 2007; Schwab et al., 2005). FTY720 administered orally was shown to inhibit skin DCs accumulation in draining lymph nodes in response to fluorescein-labeled (FITC)-dextran application (Czeloth et al., 2005). Moreover, systemic delivery of FTY720, or $S1P_1$ receptor specific agonist SEW2871, increased numbers of circulating DCs, which was associated with CCR-7 downregulation and decreased transendothelial migration (Lan et al., 2005). Daily delivery of FTY720 or S1P in the airways was shown to alleviate features of asthma in mice by affecting DC migration (Idzko et al., 2006), but whether or not this effect resulted from receptor activation; disruption of enzymatic cascades by the sphingosine analog; or from accumulation of sphingosine in the airways by rapid S1P degradation by cell surface phosphohydrolases (Le Stunff et al., 2002) was not determined.

Although we did not identify the single receptor responsible for AFD-R to induce immunosuppression, our results show that single modulation of $S1P_1$ is not sufficient to circumvent the pulmonary immune response. As AFD-R does not activate $S1P_2$, it is conceivable that single activation of $S1P_3$, $S1P_4$ or $S1P_5$ or combined activity on multiple receptors including $S1P_1$, $S1P_3$, $S1P_4$ and/or $S1P_5$

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is responsible for DC function impairment. The elucidation of the exact S1P receptors to be modulated for inhibition of pulmonary immune response is actually limited by the availability of monospecific chemical probes that are water-soluble and therefore safe for delivery in the airways.

While crucial for virus clearance, an excessive immune response can be detrimental to the host by causing damage to tissues. A balanced host immune response with adequate viral control may represent a host advantage. In our approach, the inhibitory effect of sphingosine analog on T cell accumulation in the lung was dose-responsive (Figure 1A). This result indicates that proper modulation of S1P receptors in the airways could allow the minimal T cell response required to control viruses, while preventing massive T cell proliferation and subsequent aggravation of lung injury. Indeed, even though the amount of T cells accumulated in the lung was reduced by S1P receptor modulation, these virus-specific $CD8^+$ T cells were capable of synthesizing IFN-γ and TNF-α (Figure 5B and data not shown), indicating that they could exhibit ordinary cytotoxic activity toward virus-infected cells. Thus, S1P receptor modulation has the potential to alleviate exaggerated cytokine release and T cell-mediated immunopathology, while maintaining essential CTL responses during virus infections.

In summary, we determined that sphingosine analogs were rapidly phosphorylated after delivery in the airways, and that local S1P receptor

modulation impaired function of DCs as well as cytokine release in the lungs. Accordingly, DC accumulation in the draining lymph nodes was inhibited, as well as subsequent virus-specific T cell expansion, leading to suppression of T cell accumulation in the lungs. Modulation of pulmonary immune response by S1P receptor modulators may have therapeutic implications for situations where immunopathology contributes to exacerbate disease.

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Acknowledgements

The authors would like to acknowledge the technical assistance of Nora Leaf, Megan Welch, Amol Shah, and Talya Bordin-Wosk; thank TSRI flow cytometry core facility and Bill Webb at TSRI Center for Mass Spectrometry.

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Footnotes

a) Bumsuk Hahm and David Marsolais contributed equally to the work. This is publication No 18965 from Departments of Immunology and Microbial Sciences, Infectology, Chemical Physiology, and Immunology, and The Scripps Research Institute Molecular Screening Center, The Scripps Research Institute (TSRI). This work was supported in USPHS grants AI074564 (MBAO, HR, YK, BH, KBW, DM); AI09484 (MBAO), AI05509 (HR), AI069274 (YK, YH); and NIMH-074404 (HR). YK and YH are also supported by Grants-in-aid from the Ministries of Education, Culture, Sports, Science, and Technology and of Health, Labor, and Welfare of Japan; HR is supported in part by a grant from Kyorin Pharmaceutical Company. The work of DM is supported by a fellowship from Le Fonds de la Recherche en Santé du Québec, QC, Canada.

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Molecular Pharmacology Fast Forward. Published on June 24, 2008 as DOI: 10.1124/mol.108.048769
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Figure Legends

Figure 1. Local administration of sphingosine analog AAL-R impairs influenza virus-specific CD8$^+$ T cell accumulation in the lungs following FLU-LCMV Infection. (A) Percentage of GFP$^+$ GP33/CD8$^+$ T cells was analyzed by flow cytometry 6 days post infection (dpi) in lungs of mice treated with AAL-R (0, 0.02, 0.07, 0.22, 0.67, and 2 mg/kg) one hour post infection (hpi). Pool of two experiments, n=3-6 mice per group, mean ± SEM. (B) Number of Thy1.1$^+$GP33/CD8$^+$ T cells in lungs 6 days after infection with FLU-LCMV and treatment i.t. with VEH (n=7 mice) or AAL-R (0.1 mg/kg, n=8 mice) or i.p. with VEH (n=3 mice) or AAL-R (0.1 mg/kg, n=10 mice) 1 hpi. Pool of 3 experiments, mean ± SEM, *p< 0.05. (E) Percentage of pooled CD4$^+$ and CD8$^+$ T cells in blood at 0, 0.5, 1, 3, and 6 days following i.t or i.p delivery of AAL-R (0.1 mg/kg). n=3-4 mice per group, mean ± SEM.

Figure 2. AAL-S does not inhibit virus-specific CD8$^+$ T cell response (A) Number and (B) percentage of GFP$^+$ GP33/CD8$^+$ T cells in lungs 6 days after infection with mock or FLU-LCMV and i.t. treatment with VEH, AAL-S (2 mg/kg) or AAL-R (2 mg/kg) 1 hpi. n=3 mice per group, mean ± SEM, *p< 0.05.

Figure 3. AAL-R but not AAL-S inhibits cytokine release in the lungs following FLU-LCMV infection. ELISA to detect (A) IL-6, (B) MCP-1, (C)IL-1β, and (D) TNF-α was performed 2dpi with lung homogenates obtained from mice infected i.t. with mock (CTR) (n=4) or FLU-LCMV (n=6 mice per group). Virus-

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infected mice were treated i.t. with VEH, AAL-S (0.1mg/kg), or AAL-R (0.1mg/kg) 1hpi. Mean ± SEM, *p<0.05.

Figure 4. AAL-R is rapidly phosphorylated to AFD-R following i.t. delivery. Control mice were administered with 0.3 mg/kg of AAL-R i.t. or i.p. Mice were euthanized 0.5 and 24 hours post AAL-R administration for whole body perfusion and harvest of lungs. Tissues were processed for methanol extraction and LC-MS quantification of AAL-R and AFD-R. n=3-4 mice per group, mean ± SEM, *p< 0.05.

Figure 5. AFD-R inhibits virus-specific T cell accumulation in lungs 6 days post FLU-LCMV infection. (A) Number of Thy1.1$^+$GP33/CD8$^+$ T cells in lungs, at 6dpi, of mice treated i.t. with VEH (2% cyclodextrin), AAL-R or AFD-R 1hpi. Representative of two experiments, both showed similar results, n=3-4 mice per group, mean ± SEM, *Different from VEH p< 0.05. (B) Percentages of IFN-γ or TNF-α-producing CD8$^+$ T cells were also evaluated.

Figure 6. S1P$_1$-specific receptor agonist CYM-5442 induces lymhopenia but does not inhibit virus-specific T cell response. (A) Vehicle or CYM-5442 (2 mg/kg) were delivered i.t.. Eight hours later, blood was harvested by cardiac puncture for automated analysis of total white blood cell (WBC) numbers. (B) Draining mediastinal lymph nodes (MLN) and non-draining inguinal lymph nodes (ILN) were also removed for tissue dissociation, cell counting and flow cytometric

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analysis of $CD4^+$ $CD8^+$ and $B220^+$ lymphocyte content. Shown is the summation of the three cell types for each condition. (C) Number (left) and percentage (right) of $Thy1.1^+GP33/CD8^+$ T cells in lungs 6 days after infection and treatment 1 hpi with vehicle, AAL-R (2mg/kg) and CYM-5442 (2mg/kg). n=4 mice per group; mean ± SD, *p<0.05.

Figure 7. AAL-R acts upon dendritic cells to inhibit virus-specific $CD8^+$ T cell response following FLU-LCMV infection. (A) Mice were infected with FLU-LCMV and administered with VEH or AAL-R (0.1mg/kg), i.t. or i.p. 1 hpi. MLNs were harvested 2 dpi for analyses of $CD11c^+$ cell content. n=3-4 mice per group, mean ± SEM, *p<0.05. (B) BMDCs were infected with FLU-LCMV at 3 MOI for 1h and then treated with VEH or 500 nM AAL-R in vitro for 2h. DCs (1×10^5/mouse) were administered i.t. to mice that previously received 5×10^4 $Thy1.1^+$ $GP33/CD8^+$ T cells i.v.. Six days after delivery of DCs, lungs were processed to single cell suspension for flow cytometric analysis of $Thy1.1^+$ $GP33/CD8^+$ T cells content. n=3-4 mice per group, mean ± SEM, *p<0.05. Representative of 3 different experiments obtained with similar results.

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Figure 1

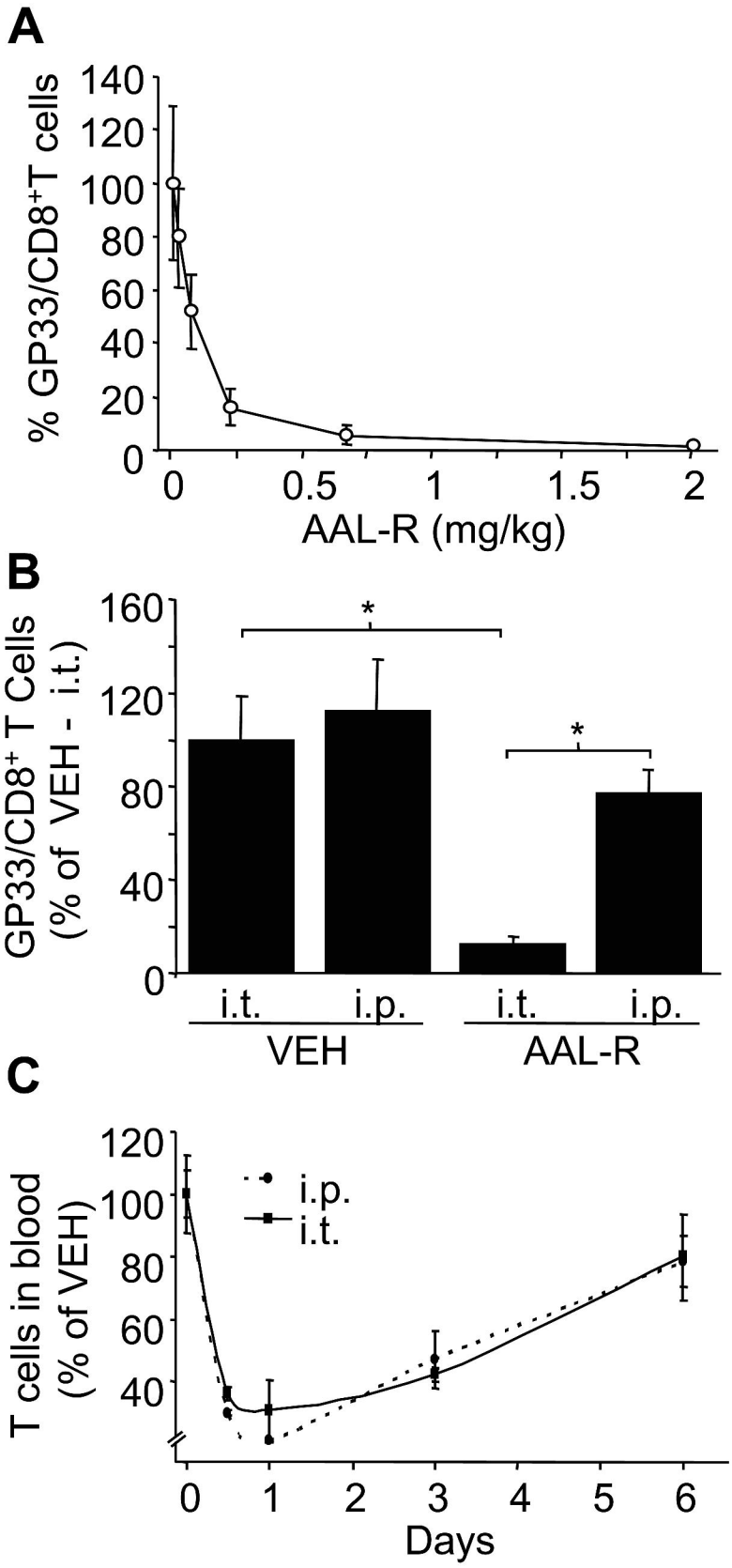

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Figure 2

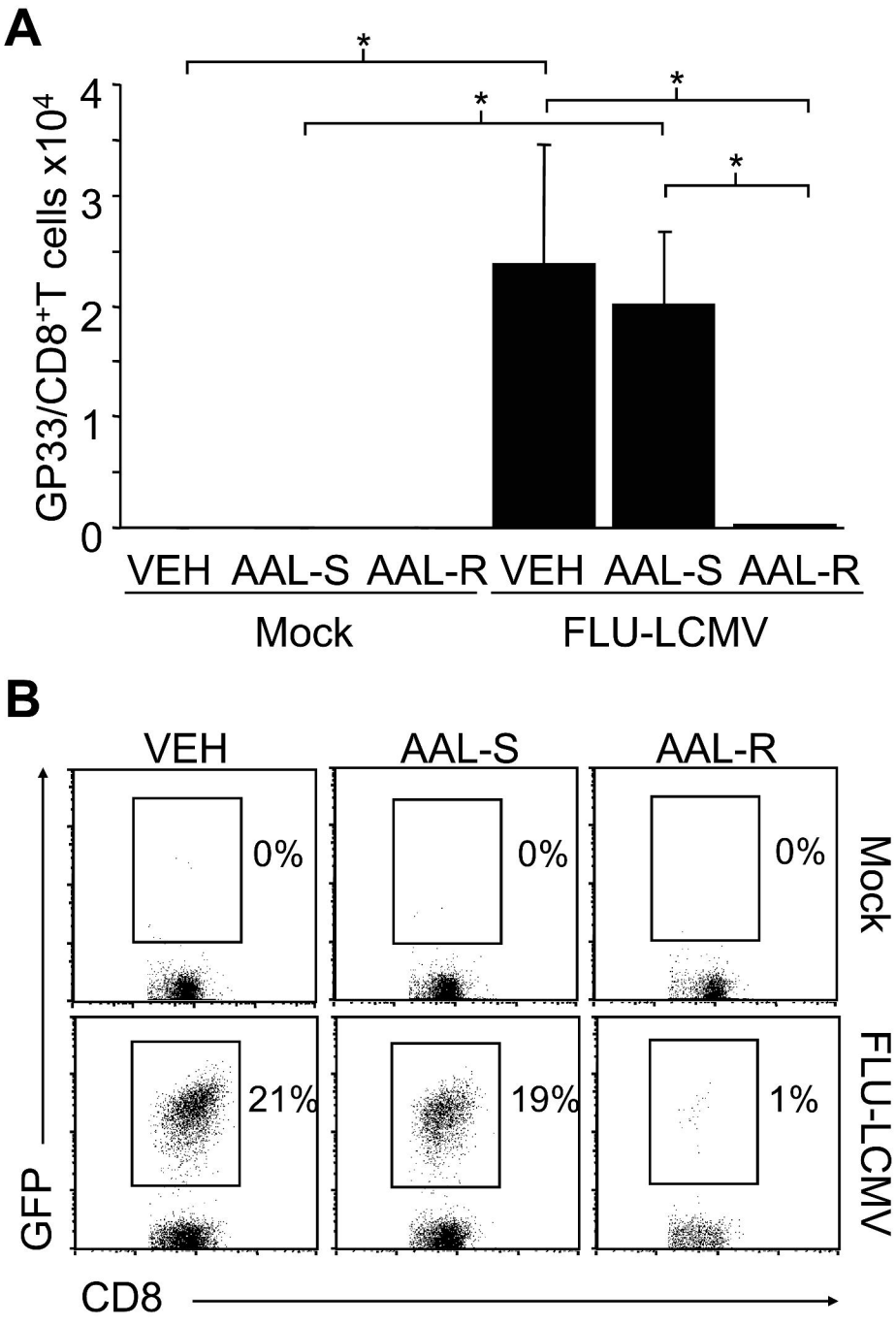

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Figure 3

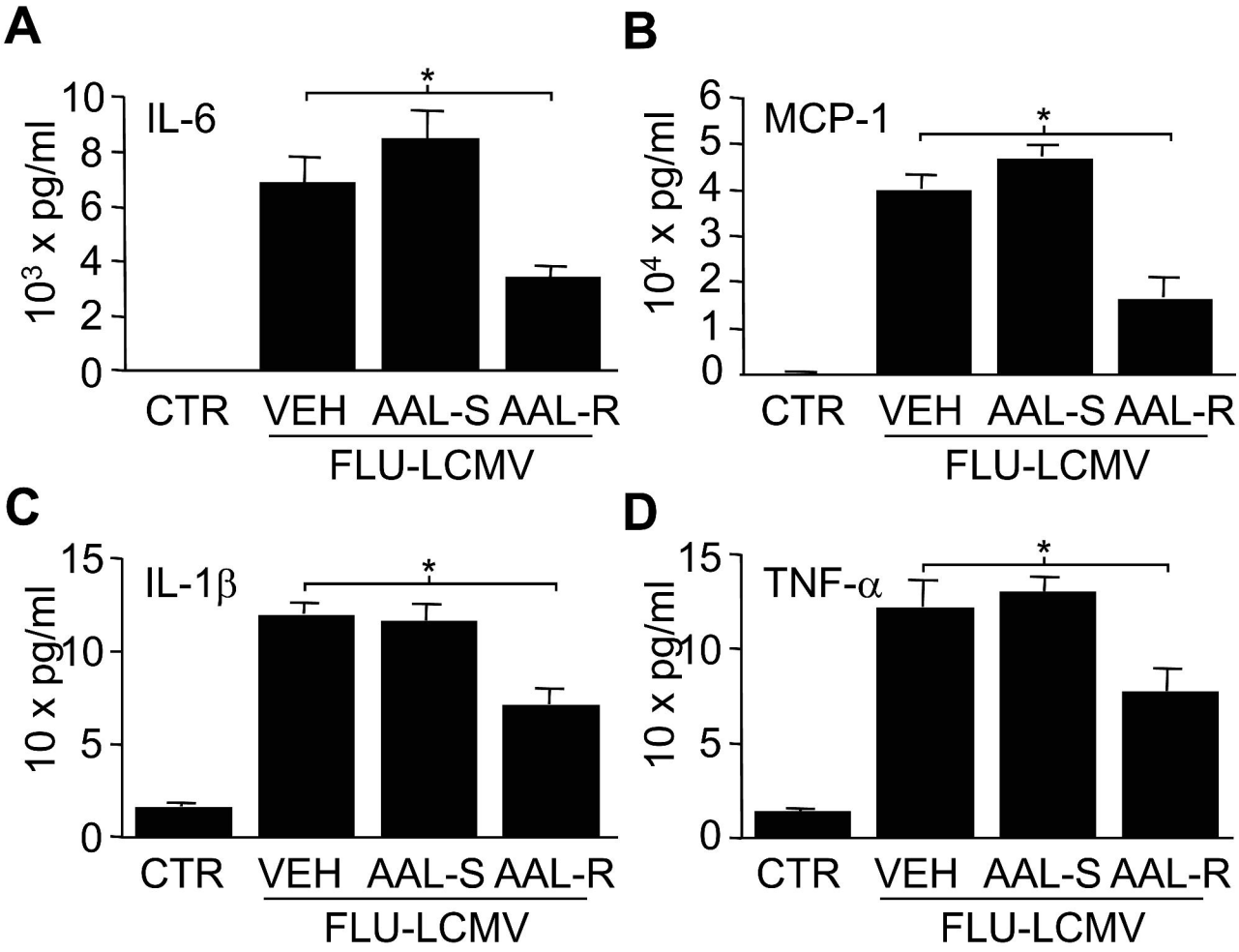

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Figure 4

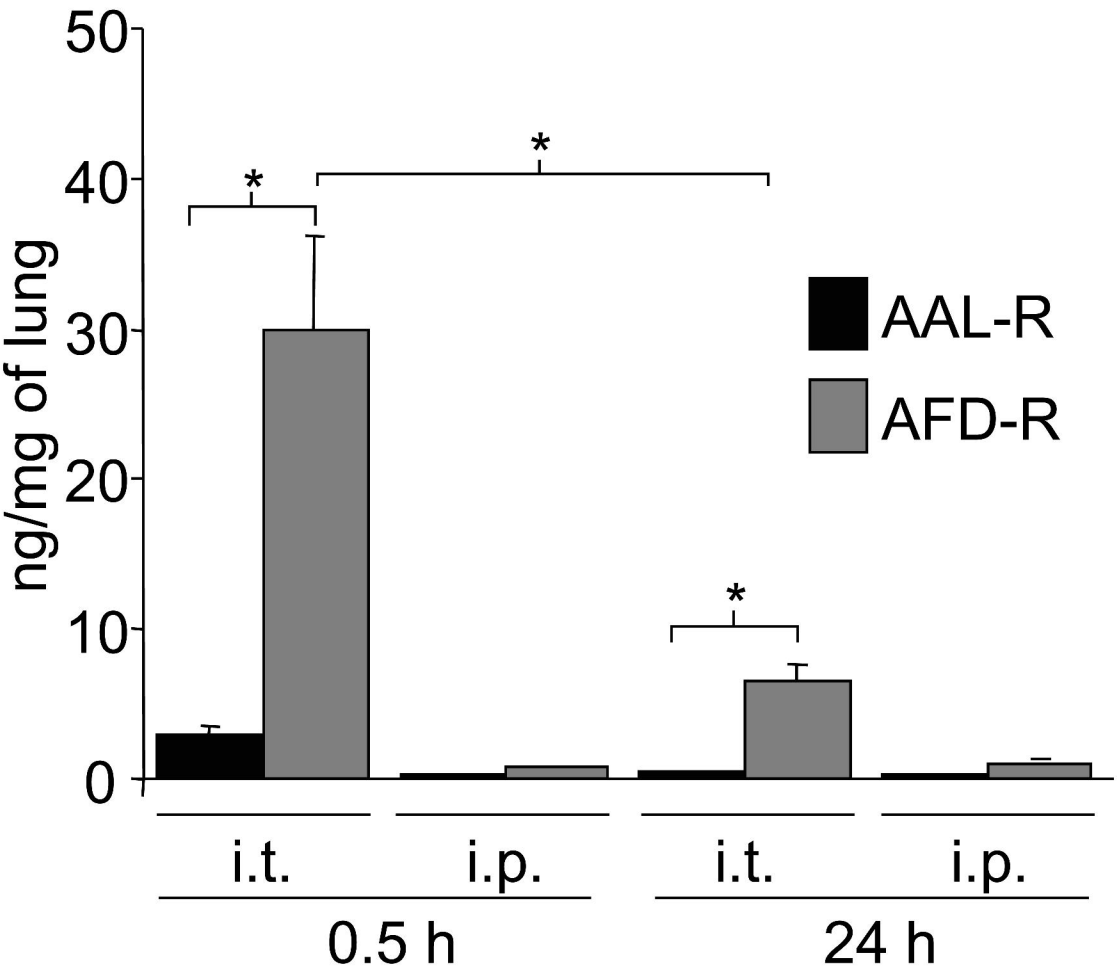

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Figure 5

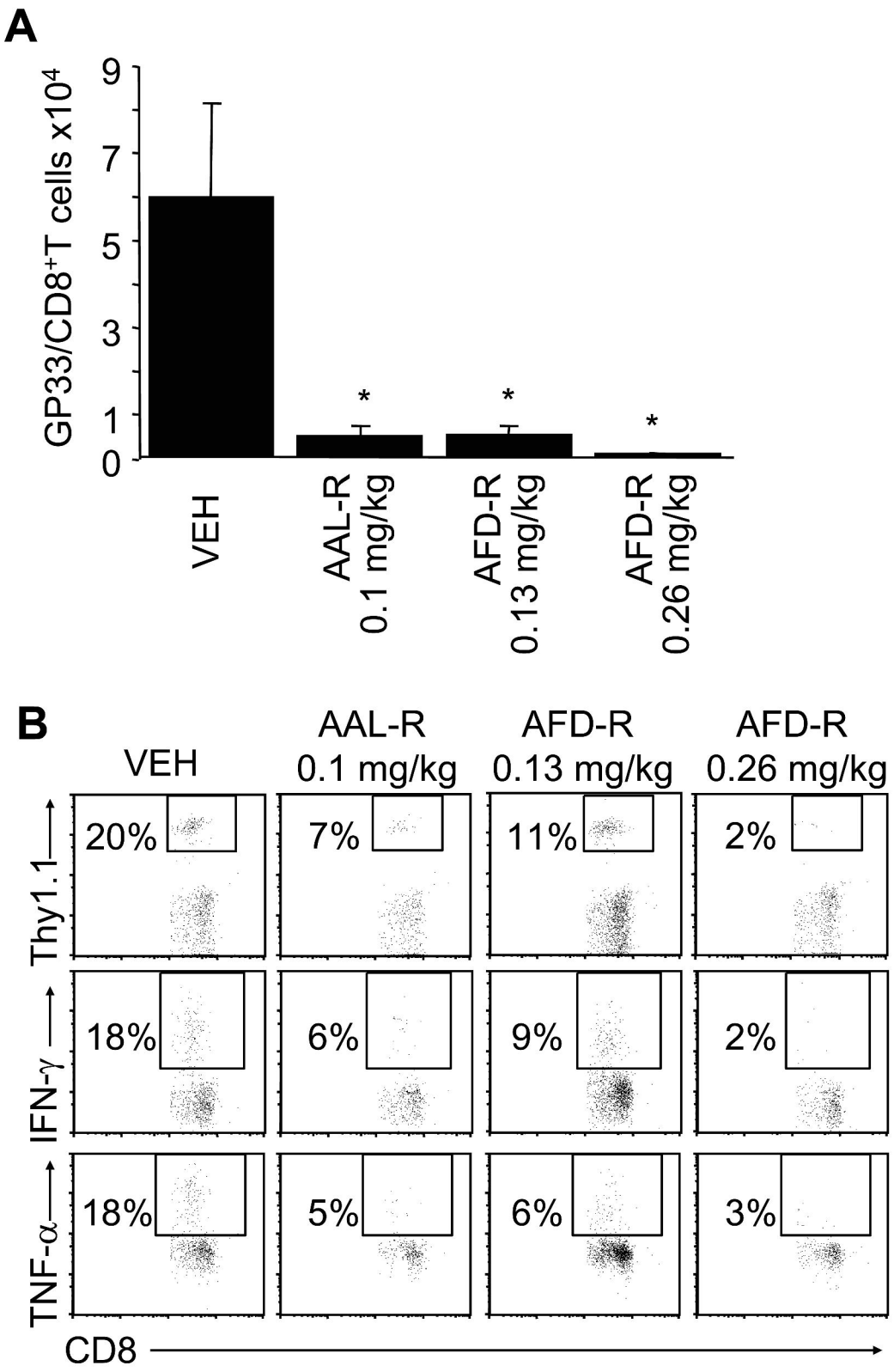

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Figure 6

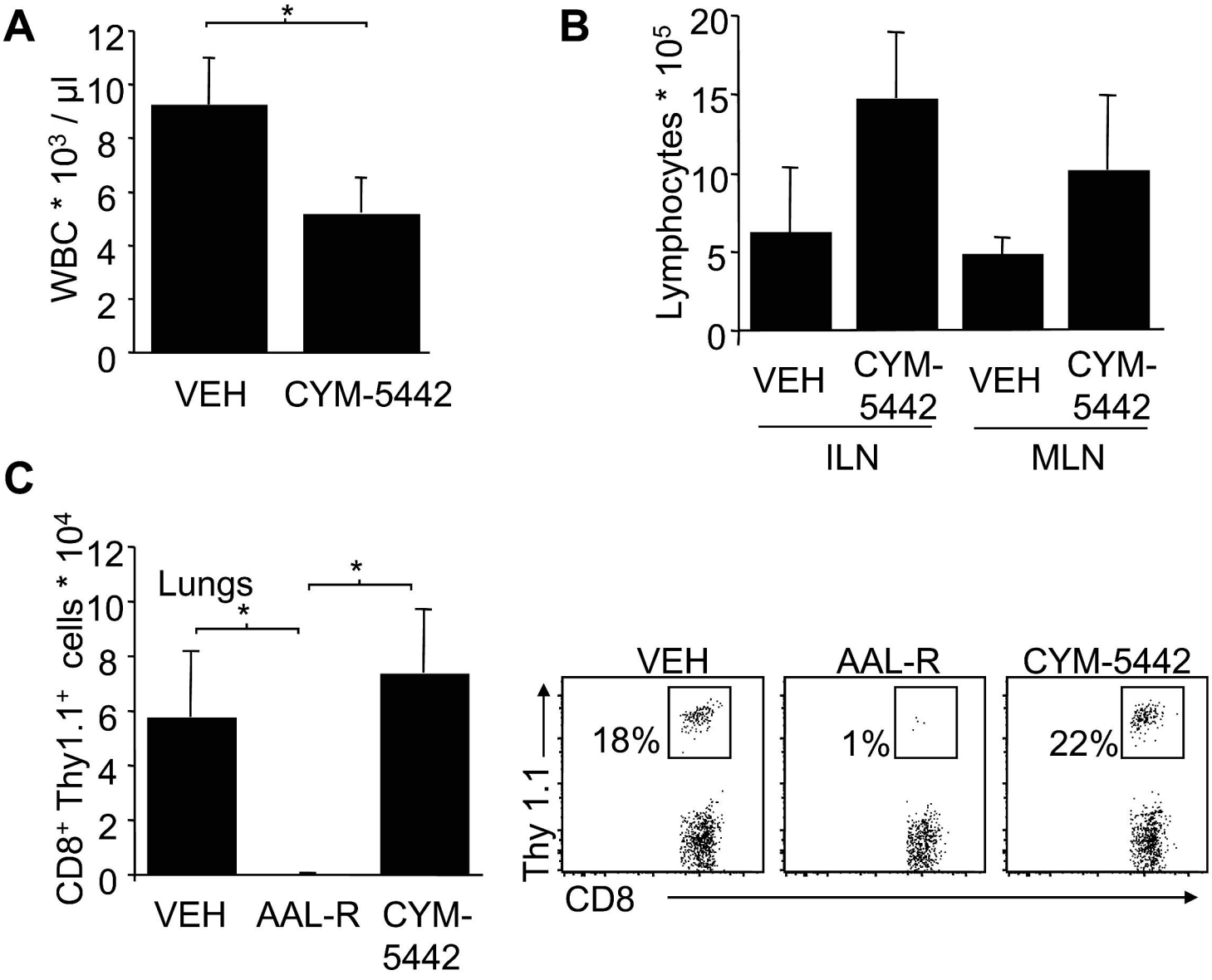

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Figure 7