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Local Not Systemic Modulation of Dendritic Cell S1P Receptors in Lung Blunts Virus-Specific Immune Responses to Influenza[S]

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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 intratracheal delivery of the chiral sphingosine analog (*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL-R) or its phosphate ester inhibits the T-cell response to influenza virus infection. In contrast, neither intraperitoneal delivery of AAL-R nor intratracheal instillation of the non-phosphorylatable stereoisomer AAL-S suppressed virus-specific T-cell response, indicating that in vivo phosphorylation of AAL-R and sphingosine 1-phosphate (S1P) receptor modulation in lungs is essential for immunomodulation. Intratracheal 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 naive lymphocyte recirculation. Accumulation of dendritic cells (DCs) in draining lymph nodes was inhibited by intratracheal but not intraperitoneal 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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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 $S1P_1$-dependent enhancement of the endothelial barrier

ABBREVIATIONS: S1P, sphingosine-1-phosphate; DC, dendritic cell; CTL, cytotoxic T lymphocyte; TNF, tumor necrosis factor; LCMV, lymphocytic choriomeningitis virus; 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; BMDC, bone marrow dendritic cell; CYM-5442, 2-(4-(5-(3,4-diethoxyphenyl)-1,2,4-oxadiazol-3-yl)-2,3-dihydro-1*H*-inden-1-ylamino)ethanol; MCP, monocyte chemoattractant protein; IL, interleukin; i.t., intratracheal; VEH, vehicle; PBS, phosphate-buffered saline; dpi, days after infection; hpi, hours after infection; FTY720, 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol; SEW2871, 5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole.

of sinus-lining endothelium (Mandala et al., 2002; Wei et al., 2005; Sanna et al., 2006). For instance, systemically and continuously delivered sphingosine analogs modulate S1P receptors (Sanna et al., 2004) after in vivo phosphorylation (Billich et al., 2003), leading to the sequestration of naive 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 of 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 (Okazaki et al., 2002; Peng et al., 2004; Bolick et al., 2005; Yopp et al., 2005; Awad et al., 2006; Ledgerwood et al., 2008) and can interfere with the function of sentinel cells such as dendritic cells (DCs), macrophages, and endothelial and epithelial cells.

The mechanisms used by sphingosine analogs to influence cellular responses are numerous. Indeed, FTY720 was shown to modulate the activity of various enzymes such as sphingosine kinase (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 pathological 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 of chemical probes and a novel system that allows monitoring influenza virus-specific T-cell responses in vivo to determine the molecular and cellular 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 intratracheal delivery and acts through S1P receptors to impair DC function and proinflammatory cytokine release in the lungs. Inhibition of virus-specific T-cell expansion acted independently of $S1P_1$ and $S1P_2$ receptor modulation and independently of naive lymphocyte recirculation. S1P receptor-mediated impairment of DC activation led to a reduction of DC accumulation in draining lymph nodes and ultimately to the blunting of influenza virus-specific T cell amplification that might be of therapeutic interest.

Materials 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 methods published previously (Kiuchi et al., 1998, 2000). Synthesis of CYM-5442 is described in details elsewhere (P. J. Gonzalez-Cabrera, S. Brown, E. Jo, M. Germana Sanna, N. Leaf, D. Marsolais, M.-T. Schaeffer, J. Chapman, M. Cameron, M. Guerrero, et al., submitted).

Virus. A mutant A/WSN/33 virus bearing the lymphocytic choriomeningitis virus (LCMV) immunodominant T-cell-specific GP33 and GP65 tandem sequence AAGGCTGTCTACAATTTTGCCACCTGTGGGGGGACGCACAAUGGGTCTTAAGGGACCCGACATTTACAAAGGAGTTTACCAATTTAAGTCAGTGGAGTTTGAT between nucleotides 145 and 146 of WSN neuraminidase 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 sequences. Virus was amplified and titrated in Madin-Darby canine kidney cells and stored at −80°C.

Cell Transfer, Virus Infection, and Administration of Compounds. Male C57BL/6 mice, (7–9 weeks old) were adoptively transferred with 5×10^4 virus-specific CD8$^+$ T cells (GFP$^+$ or Thy1.1$^+$ GP33/CD8$^+$ T cells) by injection in the tail vein. On the following day, mice received 1×10^5 PFU of FLU-LCMV i.t. under isoflurane anesthesia. One hour after infection, mice were anesthetized with an i.p. injection of 0.5 mg/g 2-2-2-tribromoethanol (Sigma, St. Louis, MO) for the intratracheal delivery of compounds (i.e., AAL-R, AAL-S, or CYM-5442) or water as vehicle (VEH), or AFD-R or 2% β-cyclodextrin in water as VEH. For some experiments, mice received intraperitoneal compounds or VEH indicated previously.

Isolation of Cells from Organs and CD8$^+$ T-Cell Enrichment. Lungs or mediastinal lymph nodes (MLNs) were harvested from PBS-perfused mice. Organs were disrupted mechanically through a 100-μm filter, and then red blood cells and lung tissue debris were removed with 0.83% ammonium chloride and 31.5% Percoll (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) 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 EDTA for 5 min before mechanical disruption. Spleens from either C57BL/6 Thy1.1$^+$D^{b}GP$_{33}$–41 TCR-tg or C57BL/6 GFP$^+$D^{b}GP$_{33}$–41 TCR-tg mice were 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., Vancouver, BC, Canada), (purity, >95%).

AAL-R, AFD-R, and Cytokine 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 described previously (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 with samples as quantification standards. For multiplexed enzyme-linked immunosorbent assay analysis of cytokines, whole lungs were homogenized in PBS and processed for analysis by Quansys Biosciences (Logan, UT).

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

DC Transfer Assay. DCs were derived from bone marrow cells (BMDCs) by incubation for 10 days with recombinant mouse granulocyte-macrophage colony-stimulating factor as described previously (Lutz et al., 1999). Cultured DCs were incubated with a multiplicity of infection of 3 of FLU-LCMV or PBS for 1 h, washed, and incubated for 2 h with VEH or AAL-R (500 nM). After being washed, 1×10^5 DCs were transferred intratracheally into C57BL/6 mice that had received 5×10^4 Thy 1.1$^+$ GP33/CD8$^+$ T cells i.v. on the previous day. Mice were

euthanized 6 days after instillation for analysis of virus-specific T cells content in lungs.

Statistical Analysis. Unless otherwise stated, bars represent means ± S.E.M., and averages were compared using a bidirectional unpaired Student's t test with a 5% significance level. Asterisk (*) was used to mark significant differences between two groups unless otherwise stated; $n \geq 3$ mice per group for all statistical analyses.

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 LCMV in its neuraminidase stalk (Neumann et al., 1999; Hahm et al., 2007) that was termed FLU-LCMV. The availability of T-cell receptor transgenic mice for these two epitopes allows for monitoring and quantification of virus-specific T-cell response in vivo as 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.

Although few or no adoptively transferred FLU-LCMV-specific $CD8^+$ T cells (GP33/$CD8^+$ T cells) can be detected in the lungs of noninfected mice, FLU-LCMV infection leads to accumulation of 2 to 5 $\times$ 10^4 FLU-LCMV-specific $CD8^+$ T cells in the lungs 6 days after infection. Intratracheal instillation of chiral sphingosine analog AAL-R 1 h after infection with recombinant FLU-LCMV virus reduced the percentage of virus-specific $CD8^+$ T cells in the lungs in a dose-dependent manner, with maximal inhibition occurring at approximately 0.3 mg/kg (Fig. 1A). Administering a single 0.1 mg/kg i.t. dose of AAL-R profoundly inhibited pulmonary accumulation of total virus-specific $CD8^+$ T cells (Fig. 1B), whereas intraperitoneal delivery of the same (Fig. 1B) or 3-fold higher amount of AAL-R (see Supplemental Data) had no significant effect on virus-specific $CD8^+$ T cell accumulation in the lungs. It is noteworthy that the kinetics of blood lymphopenia induced by local or systemic delivery of AAL-R did not differ significantly (Fig. 1C). These results indicate that modulation of pulmonary rather than systemic targets is required for T-cell suppression and that inhibition of naive 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 $S1P_{1,3-5}$ receptor agonist AFD-R. On the other hand, the 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 $\times$ 10^4 virus-specific $CD8^+$ T cells could be detected in the lungs of mice 6 days after intratracheal delivery of FLU-LCMV infection. Although intratracheal delivery of 2 mg/kg AAL-R totally suppressed virus-specific T-cell accumulation in the lungs, administration of the same dose of AAL-S had no significant effect compared with VEH treatment (Fig. 2, A and B).

AAL-S inactivity was confirmed by assessment of cytokine release 2 days after infection with FLU-LCMV virus. Compared with ambulatory control mice, interleukin (IL)-6 (~600 fold) (Fig. 3A) and monocyte chemoattractant protein (MCP)-1 (~300-fold) (Fig. 3B) were prominently elevated by FLU-LCMV infection. Both IL-6 and MCP-1 were strongly suppressed by AAL-R (52 and 59% inhibition, respectively) (Fig. 3, A and B). AAL-R also substantially reduced pulmonary content of IL-1β (47%) (Fig. 3C) and TNF-α (41%) (Fig. 3D) compared with 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 analog 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 intratracheal delivery of AAL-R by means

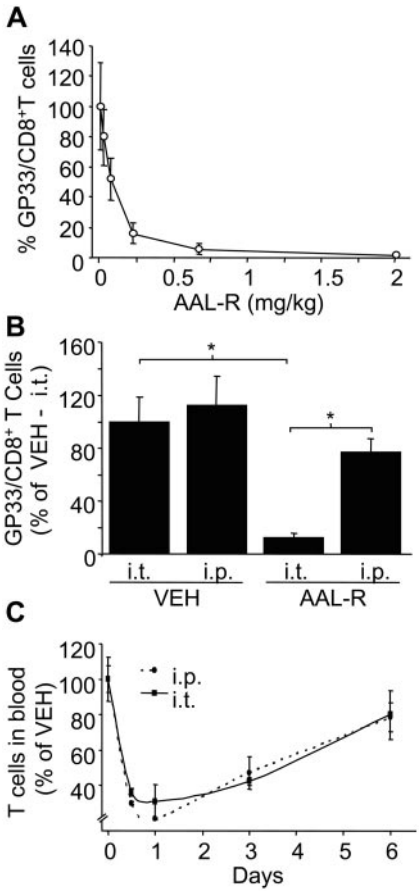

Fig. 1. Local administration of sphingosine analog AAL-R impairs influenza virus-specific $CD8^+$ T cell accumulation in the lungs after FLU-LCMV Infection. A, the percentage of GFP^+ GP33/$CD8^+$ T cells was analyzed by flow cytometry 6 days after infection (dpi) in lungs of mice treated with AAL-R (0, 0.02, 0.07, 0.22, 0.67, and 2 mg/kg) 1 h after infection (hpi). There was a pool of two experiments, n = 3–6 mice per group, mean ± S.E.M. B, the number of $Thy1.1^+$GP33/$CD8^+$ T cells in lungs 6 days after infection with FLU-LCMV and intratracheal treatment with VEH (n = 7 mice) or AAL-R (0.1 mg/kg, n = 8 mice) or intraperitoneal treatment with VEH (n = 3 mice) or AAL-R (0.1 mg/kg, n = 10 mice) 1 hpi. Pool of three experiments, mean ± S.E.M., *, $p < 0.05$. E, the percentage of pooled $CD4^+$ and $CD8^+$ T cells in blood at 0, 0.5, 1, 3, and 6 days after intratracheal or intraperitoneal delivery of AAL-R (0.1 mg/kg); n = 3–4 mice per group, mean ± S.E.M.

of liquid chromatography-mass spectrometry (Fig. 4). After intratracheal 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 h after 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 30 min after administration compared with intraperitoneal delivery. Pulmonary AFD-R content was diminished by 80% after 24 h, highlighting the transient nature of the treatment (Fig. 4). The decreased compound concentration in the lungs probably reflects redistribution to the bloodstream and the interstitial fluids, which is in keeping with the systemic lymphopenia observed (Fig. 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 suppression of T-cell accumulation

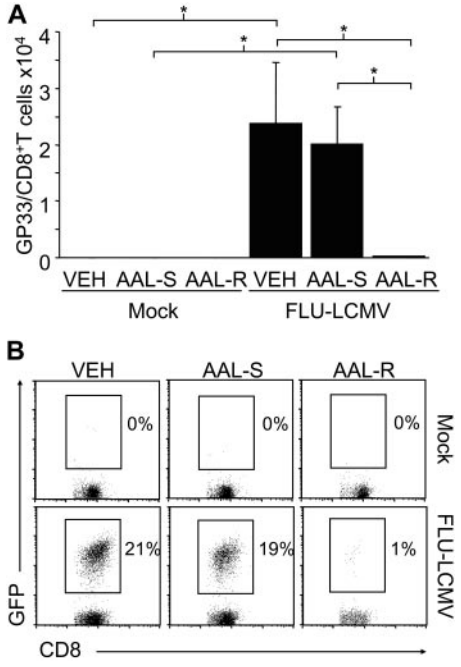

Fig. 2. AAL-S does not inhibit the virus-specific CD8$^+$ T cell response. Number (A) and percentage (B) of GFP$^+$ GP33/CD8$^+$ T cells in lungs 6 days after infection with mock or FLU-LCMV and intratracheal treatment with VEH, AAL-S (2 mg/kg), or AAL-R (2 mg/kg) 1 hpi; $n = 3$ mice per group, mean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

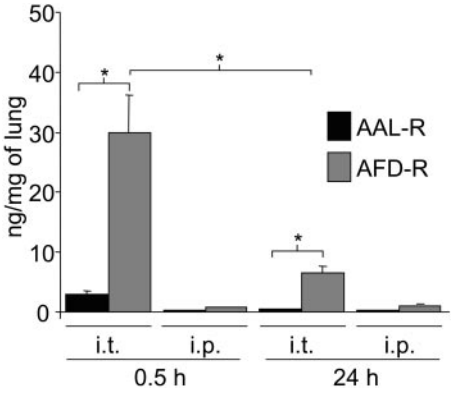

Fig. 4. AAL-R is rapidly phosphorylated to AFD-R after intratracheal delivery. Control mice were administered with 0.3 mg/kg AAL-R i.t. or i.p. Mice were euthanized 0.5 and 24 h after AAL-R administration for whole-body perfusion and harvest of lungs. Tissues were processed for methanol extraction and liquid chromatography/mass spectrometry quantification of AAL-R and AFD-R; $n = 3$–4 mice per group, mean ± S.E.M., *, $p < 0.05$.

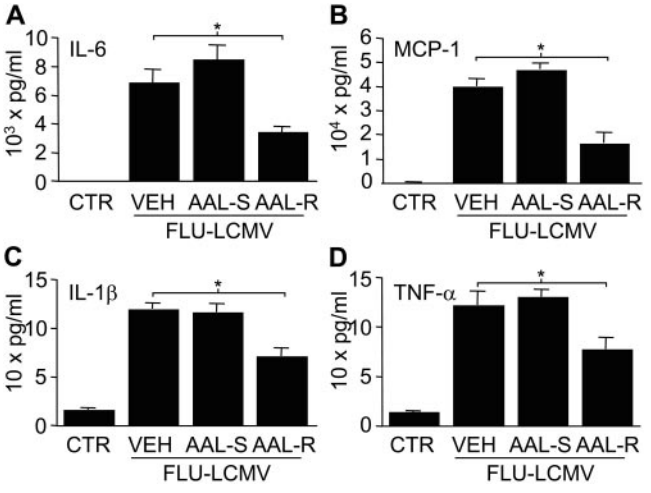

Fig. 3. AAL-R but not AAL-S inhibits cytokine release in the lungs after FLU-LCMV infection. Enzyme-linked immunosorbent assay to detect IL-6 (A), MCP-1 (B), IL-1β (C), and TNF-α (D) was performed 2 dpi with lung homogenates obtained from mice infected intratracheally with mock (CTR) ($n = 4$) or FLU-LCMV ($n = 6$ mice per group). Virus-infected mice were treated intratracheally with VEH, AAL-S (0.1 mg/kg), or AAL-R (0.1 mg/kg) 1 hpi. Mean ± S.E.M., *, $p < 0.05$.

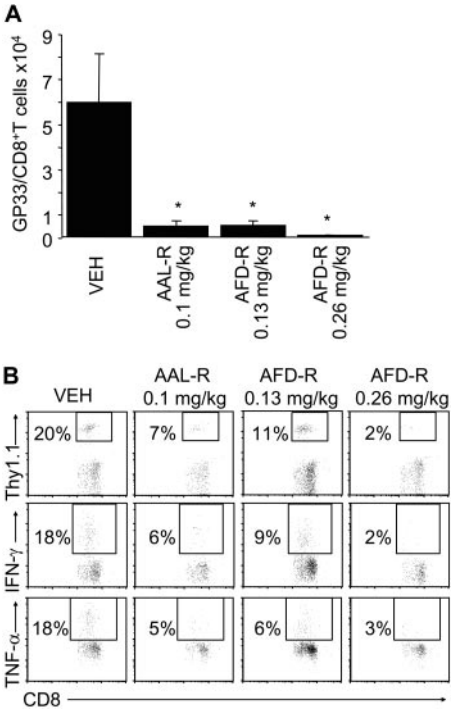

Fig. 5. AFD-R inhibits virus-specific T-cell accumulation in lungs 6 days after FLU-LCMV infection. A, the number of Thy1.1$^+$GP33/CD8$^+$ T cells in lungs, at 6 dpi, of mice treated intratracheally with VEH (2% cyclodextrin), AAL-R, or AFD-R 1 hpi. Representative of two experiments, and both showed similar results; $n = 3$–4 mice per group, mean ± S.E.M. *, different from VEH $p < 0.05$. B, percentages of IFN-γ or TNF-α-producing CD8$^+$ T cells were also evaluated.

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 intratracheally after 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 (Fig. 5, A and B), indicating that immunosuppression did not require intracellular activities of either AAL-R or AFD-R (Don et al., 2007). It is noteworthy that virus-specific CD8$^+$ T cells were barely detectable in the lungs of mice treated with 0.26 mg/kg AFD-R i.t. 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 after infection, we used a recently developed water-soluble S1P$_1$-specific receptor agonist. CYM-5442 induces systemic lymphopenia (Fig. 6A) and sequestration of T lymphocytes in nondraining lymph nodes 8 h after intratracheal delivery, showing in vivo effectiveness (Fig. 6B). In accordance with documented lymphopenic response induced by S1P$_1$-specific agonists (Sanna et al., 2004; Pan et al., 2006), a single intraperitoneal injection of CYM-5442 induced lymphopenia for least 18 h (data not shown). Despite systemic effects on naive lymphocyte recirculation, intratracheal delivery of CYM-5442 for up to 2 mg/kg i.t. (Fig. 6C; data not shown) did not impair the percentage or total number of virus-specific CD8$^+$ T cells in the lungs 6 days after FLU-LCMV infection.

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 after infection with FLU-LCMV, indicating that AAL-R inhibited clonal expansion of T cells upon infection (data not shown). Intratracheal delivery of AAL-R did not reduce the numbers and viability of DCs in the lungs (data not shown) but inhibited their accumulation in the MLNs (Fig. 7A). It is interesting that intraperitoneal delivery of AAL-R did not affect the accumulation of DCs in MLNs (Fig. 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 after infection, whereas uninfected DCs did not (Fig. 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% (Fig. 7B). BMDCs have the ability to phosphorylate AAL-R in vitro, because we determined that 83 $\pm$ 0.9% of the species recovered from pooled cells and culture supernatant 4 h after addition of AAL-R to BMDCs were the phosphate ester, which further supports that sphingosine analog could directly modulate DC

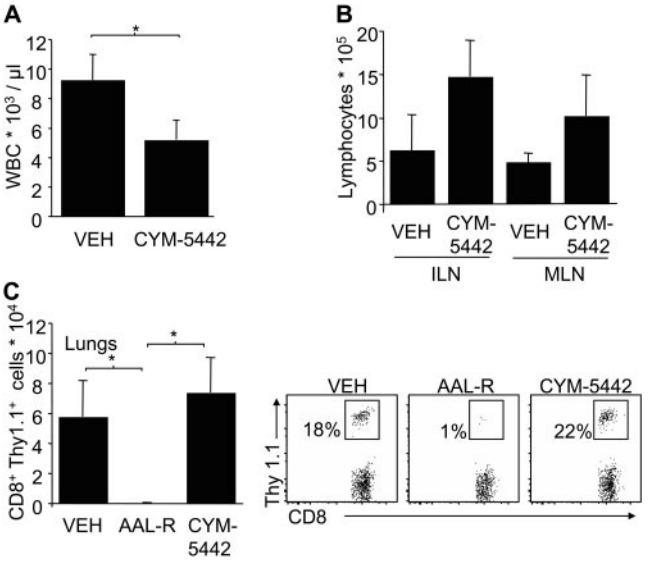

Fig. 6. S1P$_1$-specific receptor agonist CYM-5442 induces blood lymphopenia but does not inhibit virus-specific T-cell response. A, vehicle or CYM-5442 (2 mg/kg) were delivered intratracheally. Eight hours later, blood was harvested by cardiac puncture for automated analysis of total white blood cell (WBC) numbers. B, draining MLNs and nondraining inguinal lymph nodes (ILNs) were also removed for tissue dissociation, cell counting, and flow cytometric 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 (2 mg/kg) and CYM-5442 (2 mg/kg); $n = 4$ mice per group; mean $\pm$ S.D., $*$, $p < 0.05$.

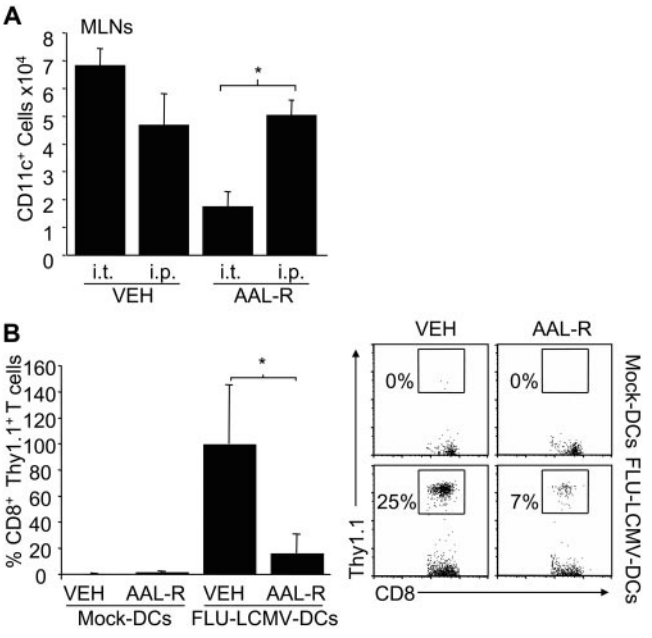

Fig. 7. AAL-R acts on dendritic cells to inhibit virus-specific CD8$^+$ T-cell response after FLU-LCMV infection. A, mice were infected with FLU-LCMV and administered with VEH or AAL-R (0.1 mg/kg), i.t. or i.p. 1 hpi. MLNs were harvested 2 dpi for analysis of CD11c$^+$ cell content; $n = 3$ to 4 mice per group, mean $\pm$ S.E.M., $*$, $p < 0.05$. B, BMDCs were infected with FLU-LCMV at a multiplicity of infection of 3 for 1 h and then treated with VEH or 500 nM AAL-R in vitro for 2 h. DCs (1×10^5/mouse) were administered intratracheally 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 into a single cell suspension for flow cytometric analysis of Thy1.1$^+$ GP33/CD8$^+$ T-cell content; $n = 3$ to 4 mice per group, mean $\pm$ S.E.M., $*$, $p < 0.05$. Representative of three different experiments obtained with similar results.

activities of T-cell stimulation upon influenza virus infection by acting on S1P receptors.

Discussion

In this report, we used chemical probes to delineate the in vivo molecular mechanism used 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 naive lymphocyte sequestration in the secondary lymphoid organs but involves intrapulmonary accumulation of S1P receptor modulatory molecules 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 are currently available regarding the modulatory potential and mechanisms of sphingosine analogs in response to pathogenic viral infections. It was shown previously 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 sufficient to inhibit proinflammatory cytokine release and virus-specific T-cell response 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 intraperitoneal delivery of AAL-R to induce the 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 nonspecifically inducing the sequestration of normally expanding lymphocytes in secondary lymphoid organs as shown previously 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 after 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 or AFD-R could induce apoptosis of selected Jurkat cells in vitro (Don et al., 2007). Indeed, whereas both AAL-R and AAL-S but not AFD-R can penetrate the cells, only AAL-R can be phosphorylated intracellularly and therefore induce cell death. 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 four 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 compared with sphingosine kinase 2 (Billich et al., 2003; Zemann et al., 2006). Despite 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 h (Fig. 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 nonphosphorylatable 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 made more complex by differential distribution of the five 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 up-regulated on DCs upon maturation (Czeloth et al., 2005), and treatment with nanomolar concentrations of phosphorylated sphingosine analog FTY720-P interferes with DC migration toward S1P and with S1P-induced phagocytosis in vitro (Maeda et al., 2007). However, most in vivo studies were performed with nonphosphorylated FTY720, which can act independently of cell-surface S1P receptor modulation (Bandhuvula et al., 2005; Schwab et al., 2005; Don et al., 2007; Dudek et al., 2007). FTY720 administered orally was shown to inhibit skin DC accumulation in draining lymph nodes in response to fluorescein isothiocyanate-labeled dextran application (Czeloth et al., 2005). Moreover, systemic delivery of FTY720, or $S1P_1$ receptor-specific agonist SEW2871, increased the numbers of circulating DCs, which was associated with chemokine receptor CCR7 down-regulation 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 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. Because 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 $S1P_5$ 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.

Although 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 (Fig. 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 (Fig. 5B; 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 control 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 the function of DCs and cytokine release in the lungs. Therefore, 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 in which immunopathology contributes to exacerbating a disease.

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