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Sphingosine Analogue AAL-R Increases TLR7-Mediated Dendritic Cell Responses via p38 and Type I IFN Signaling Pathways

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Sphingosine analogues display immunosuppressive activities and thus have therapeutic potential in the treatment of autoimmune diseases. In this study, we investigated the effects of the sphingosine analogue AAL-R (FTY720 derivative) on dendritic cell (DC) response upon TLR stimulation. Unlike its known immunosuppressive activity, AAL-R increased TLR7-mediated DC responses by elevating the levels of MHC class I and costimulatory molecules and type I IFN expression and by enhancing the capacity of DCs to induce $CD8^+$ T cell proliferation. Importantly, the stimulatory activity of AAL-R was dependent on type I IFN signaling, as type I IFN receptor-deficient DCs failed to respond to AAL-R. Also, AAL-R activated p38 MAPK to increase type I IFN synthesis and TLR7-mediated DC maturation. These findings enhance our understanding of sphingosine regulation of the host immune system, in particular upon pathogenic infections. ***The Journal of Immunology*****, 2012, 188: 4759–4768.**

Sphingosine 1-phosphate (S1P) and the synthetic analogues of sphingosine have diverse immunomodulatory functions with therapeutic potential (1–5). One sphingosine analogue, FTY720, has been proved to prolong allograft survival (6), abrogate experimental asthma (7, 8), and alleviate autoimmune diseases such as rheumatoid arthritis (9) and type I diabetes (10). Indeed, FTY720 was recently approved for the treatment of multiple sclerosis by the U.S. Food and Drug Administration as an orally administered drug (11, 12). In addition, the hydroxyl group-eliminated derivative of FTY720, (*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL-R), was shown to relieve influenza virus-induced immune pathology (13–15). AAL-R was demonstrated to regulate the expression of inflammatory cytokines in mice, although it did not significantly alter influenza virus propagation (14).

Sphingosine is metabolized by sphingosine kinase 1 (SphK1) or sphingosine kinase 2 (SphK2) to become S1P (16). The S1P may activate certain cellular signal inside cells or be secreted and bind its cognate receptors (S1PR 1–5) to trigger the receptor-mediated signaling pathways (1). FTY720 and AAL-R are phosphorylated by SphK2, but not by SphK1, and could activate S1PR1, S1PR 3–5, but not S1PR2 (17–21). These analogues were shown to act as potent agonists on the S1P receptors (18, 21, 22), although they could downregulate S1PR1 on T cells (23, 24).

The immunosuppressive effects of sphingosine analogues are mediated by diverse mechanisms. For instance, FTY720 was reported to hamper migration of mature murine dendritic cells (DCs) toward S1P (25) and impair the immunostimulatory capacity of human monocyte-derived DCs (26). In addition, FTY720 inhibits lymphocyte emigration from lymphoid organs, resulting in transient lymphopenia in the bloodstream (22, 23). Of note, AAL-R was shown to be a more potent inducer of lymphopenia than FTY720, presumably due to an increased phosphorylation rate (27).

DCs are the most potent APCs, as they efficiently transmit a pathogen's danger signal to pathogen-specific T lymphocytes (28, 29). DCs sense pathogen-associated molecular patterns (PAMPs) through highly conserved pattern-recognizing receptors (PRRs). TLRs are the best-characterized class of PRRs in mammalian species. To date, at least 10 and 12 functional TLRs have been identified in humans and mice, respectively (30). Among these, TLR7 is activated by ssRNAs and mainly located in endosomal compartments of immune cells including DCs and B lymphocytes (30, 31). The stimulation of DCs with TLR7 ligand induces DC maturation and the production of cytokines including type I IFNs (32–35), resulting in the activation of host immune responses. Importantly, type I IFNs have a prominent role in host immunity against viral infections by triggering innate anti-viral responses and promoting DC maturation and T cell activation (36–38). Therefore, activation of the immune system by TLR7 ligation could be crucial for control of diseases induced by ssRNA viral infections.

Although sphingosine analogues are known to have numerous immunomodulatory functions, their roles in DCs, which are stimulated with PAMPs, have been poorly explored. In this study, we provide the first evidence to our knowledge that a sphingosine analogue enhances DC responses through p38 and type I IFN signaling upon TLR7 stimulation.

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Abbreviations used in this article: AAL-R, hydroxyl group-eliminated derivative of FTY720 [(*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol]; BM, bone marrow; DC, dendritic cell; GP33, $GP_{33–41}$; IFNAR, type I IFN receptor; ISG15, IFN-stimulated gene 15; ko, knockout; Lox, loxoribine; MFI, mean fluorescence intensity; MHC-I, MHC class I; MHC-II, MHC class II; PAMP, pathogen-associated molecular pattern; PI, propidium iodide; poly(I:C), polyriboinosinic polyribocytidylic acid; PRR, pattern-recognizing receptor; qPCR, real-time quantitative PCR; S1P, sphingosine 1-phosphate; SphK1, sphingosine kinase 1; SphK2, sphingosine kinase 2; tg, transgenic; TRIF, TIR-domain–containing adapter-inducing IFN-β; Wt, wild-type.

Materials and Methods

Mice

C57BL/6 and C57BL/6-TLR7 knockout (ko) mice, which were purchased from The Jackson Laboratory (39), C57BL/6-Thy1.1$^+$D^{b}GP$_{33–41}$ TCR transgenic (tg) mice (40), and C57BL/6-IFNAR ko mice (41) were used. Mice were bred and maintained in a closed breeding facility according to institutional guidelines and with protocols approved by the Animal Care and Use Committee of University of Missouri-Columbia.

Dendritic cells

DCs were derived from bone marrow (BM) cells by culture with RPMI 1640 medium containing 20 ng/ml recombinant mouse GM-CSF (PeproTech) for 10 d (42). DCs were treated with TLR agonists and then treated with AAL-R (1 μM) unless indicated differently. DC maturation was assessed at 1 or 2 d after the treatment with TLR ligands.

TLR agonists, sphingosine analogues, and specific inhibitors of signaling pathways

Peptidoglycan (Sigma-Aldrich), a synthetic analogue of dsRNA, polyriboinosinic polyribocytidylic acid [poly(I:C)] (InvivoGen), LPS (Alexis Biochemicals), and CpG DNA (ODN 1826; InvivoGen) were used to stimulate TLR2, TLR3, TLR4, and TLR9 on DCs, respectively. To stimulate TLR7 specifically, we have used loxoribine, a guanosine analogue derivatized at position N^7 and C^8, of which recognition is restricted to TLR7 (43–45), and imiquimod, an imidazoquinoline amine analogue to guanosine (46). FTY720 was purchased from Cayman Chemicals, and AAL-R and AAL-S were gifts from Dr. Hugh Rosen (The Scripps Research Institute). Purified water and DMSO were used as solvent controls (vehicle) for AAL-R/AAL-S and FTY720, respectively. The specific inhibitors for p38 MAPK (SB203580) and MEK/MAPK (PD98059 and U0126) were purchased from Calbiochem.

In vitro T cell proliferation assay

Lymphocytic choriomeningitis virus epitope GP$_{33–41}$ (GP33)-specific CD8$^+$ T cells were purified from lymphocytic choriomeningitis virus epitope GP33-specific TCR tg mice by EasySep (Stemcell Technologies) and stained with CFSE (Invitrogen). Wild-type (Wt) or type I IFN receptor (IFNAR)-deficient DCs were treated with TLR7 agonist loxoribine in the presence of GP33 peptide for 2 h and then treated with vehicle (a solvent control) or AAL-R for 2 or 6 h. GP33-specific CD8$^+$ T cells were mixed with DCs at a DC/T cell ratio of 1:10. After 3 d, the proliferation of Ag-specific T cells was evaluated by the decrease in CFSE fluorescence observed in flow cytometric analyses.

Real-time PCR

Total cellular RNA was purified by using TRI-reagent (Sigma-Aldrich) according to the manufacturer's description and treated with DNase I (Fermentas) to remove contaminating DNAs. Total RNA was reverse-transcribed, and then cDNA was analyzed by real-time quantitative PCR (qPCR) using gene-specific primer sets. Primers for IFN-β (5′-CTC CAG CTC CAA GAA AGG ACG-3′, 5′-ATC TCT TGG ATG GCA AAG GCA-3′) and GAPDH (5′-TCA CCA CCA TGG AGA AGG-3′, 5′-GAT AAG CAG TTG GTG GTG CA-3′) were used. The qPCR was performed with SYBR Green I chemistry using an ABI 7900 HT real-time PCR instrument. The authenticity of the PCR products was verified by melting curve analysis. cDNA quantities were normalized to GAPDH cDNA quantities measured in the same samples.

Western blot analysis

Specific Abs against α-tubulin, STAT1, p-STAT1, STAT2, p-STAT2, ERK, p-ERK, p38, p-p38, p-MSK1, p-AKT, p-MEK, and IFN-stimulated gene 15 (ISG15) were purchased from Cell Signaling Technology, Upstate, or Santa Cruz Biotechnology. Total proteins were extracted with RIPA buffer supplemented with inhibitors blocking proteases and phosphatases and then normalized using a Bradford assay. The protein samples (20 μg each) were resolved on a 10 or 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Whatman). Membrane-bound Abs were detected with ECL (Pierce).

Flow cytometric analysis

Abs used in this study were specific for murine CD11c, MHC class I (MHC-I; H-2K^b), MHC class II (MHC-II; I-A^b), CD80 (B7-1), CD86 (B7-2), CD40, CD8α, CD90.1 (Thy1.1), TNF-α, and IL-12 (BD Pharmingen or eBioscience). For the intracellular cytokine staining, cells were treated with 5 μg/ml brefeldin A (Sigma) for 6 h and then stained with Abs. Apoptotic cell death was detected by using an annexin V–FITC apoptosis detection kit (BD Pharmingen). Briefly, cells (1×10^5) were washed twice with annexin V-binding buffer and then incubated with annexin V–FITC and propidium iodide (PI) for 15 min at room temperature in the dark. Data were collected on a CyAn ADP flow cytometer (Beckman Coulter) and analyzed with FlowJo (Tree Star) software.

Statistical analysis

All error bars represent mean ± SEM, and averages were compared using a bidirectional, unpaired Student *t* test.

Results

Sphingosine analogue increases DC activation upon TLR7 stimulation

To investigate whether the sphingosine analogue AAL-R regulates DC maturation upon TLR stimulation, DCs were challenged with diverse TLR agonists. Peptidoglycan, poly(I:C), LPS, loxoribine (Lox), or CpG were exogenously supplied to DCs to stimulate TLR 2, 3, 4, 7, or 9, respectively, in the absence or presence of AAL-R for 2 d (Supplemental Fig. 1A). MHC-I expression on DCs was then analyzed by flow cytometry. The treatment with LPS (200 ng/ml) alone markedly increased MHC-I surface expression on DCs, indicative of phenotypic DC maturation. However, AAL-R strongly blocked MHC-I upregulation on LPS-activated DCs. Upon treatment with even lower concentrations (10 or 50 ng/ml) of LPS, MHC-I upregulation was strongly inhibited by AAL-R (Supplemental Fig. 1B). Also, AAL-R inhibited MHC-I expression on poly(I:C)-stimulated DCs (Supplemental Fig. 1A). These observations indicate its previously known immunosuppressive activity (13, 14). In contrast, concomitant AAL-R treatment upregulated MHC-I surface expression when DCs were stimulated with peptidoglycan, Lox, or CpG (Supplemental Fig. 1A). Therefore, AAL-R represses TLR3- or TLR4-induced MHC-I expression, whereas the analogue appears to cooperate with TLR2, TLR7, or TLR9 ligands for MHC-I upregulation on maturing DCs. These results led us to conclude that the sphingosine analogue AAL-R differentially regulates TLR-mediated DC maturation, and the type of TLRs activated is critical for the outcome.

Because TLR7 recognizes specific ssRNA molecules (39, 47, 48), TLR7-mediated DC responses can induce potent host immune responses against pathogens including RNA viruses. Therefore, we focused on the effect of AAL-R on TLR7-stimulated DCs by using a TLR7-specific ligand and further evaluated its stimulatory activity. AAL-R significantly increased MHC-I surface expression [mean fluorescence intensity (MFI) ± SEM: 127 ± 4.1 → 232 ± 1.6, *left panel* of Fig. 1A] on DCs upon Lox treatment compared with Lox treatment with vehicle in a dose-dependent manner (Supplemental Fig. 1C). Consistent with the increase of MHC-I, when DCs were challenged with Lox, AAL-R enhanced surface expression of costimulatory molecules B7-1 (MFI ± SEM: 90 ± 0.1 → 119 ± 4.8, Fig. 1B), B7-2 (338 ± 8 → 410 ± 13, Fig. 1C), and CD40 (37 ± 1.6 → 58 ± 2.3, Fig. 1D) on DCs without causing apoptotic cell death (annexin V$^-$/PI$^-$: Lox plus vehicle, 65%; Lox plus AAL-R, 69%, Fig. 1E). However, treatment with AAL-R alone scarcely changed the surface expression of these molecules (Fig. 1A–D). These data corroborate the stimulatory effects of AAL-R on the phenotypic maturation of TLR7-stimulated DCs. Further, AAL-R enhanced DC ability to synthesize inflammatory cytokines TNF-α (2.7% → 6.9%, Fig. 2A) and IL-12 (2.1% → 5.2%, Fig. 2B) upon Lox treatment compared with Lox plus vehicle treatment, although AAL-R alone did not induce the synthesis of those cytokines in DCs (Fig. 2A, 2B). The production of the secreted soluble form of TNF-α was assessed by ELISA and shown to be increased by AAL-R at 6 or 12 h (Fig. 2C) after Lox treatment.

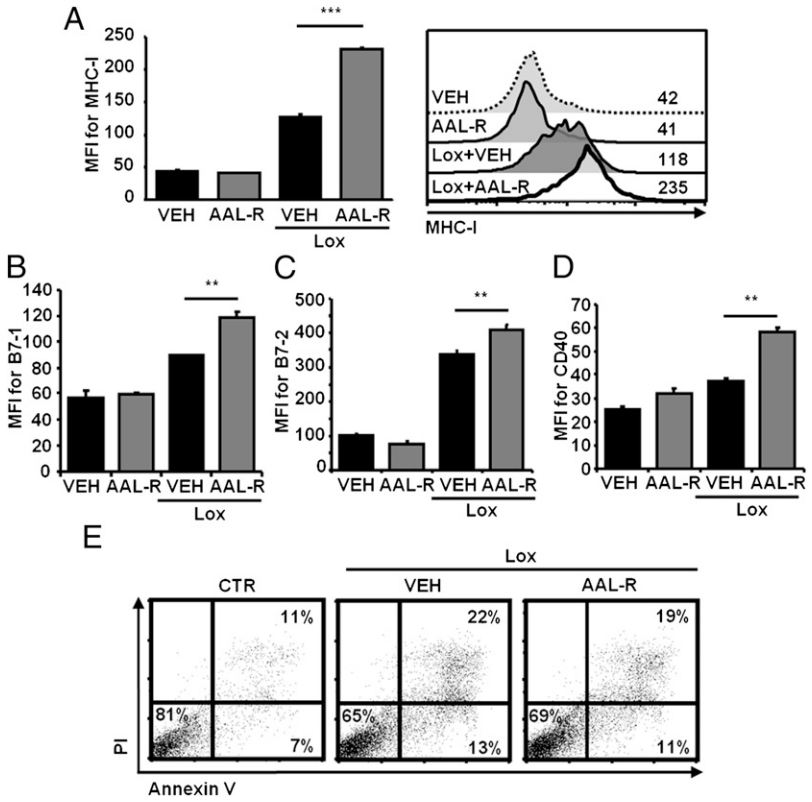

FIGURE 1. AAL-R increases DC maturation upon Lox treatment. DCs were left untreated (control) or treated with Lox (TLR7L, 1 mM); these cells were supplied with vehicle (a solvent control for AAL-R) or AAL-R (1 μM). At 2 d posttreatment, $CD11c^+$ cells were analyzed for the surface expression of MHC-I (**A**), B7-1 (**B**), B7-2 (**C**), and CD40 (**D**) or stained with annexin V/PI (**E**) and then analyzed by flow cytometry. Graphs represent the average of MFI and SEM of data obtained from each condition (n = 3/group). The data shown are representative of two to five independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$. CTR, control; VEH, vehicle.

DCs potently prime naive $CD8^+$ T cells, which play a critical role in eliminating virus-infected cells. Thus, we determined if AAL-R enhances the ability of DCs to promote $CD8^+$ T cell proliferation upon TLR7 stimulation. DCs were pulsed with GP33 peptide in the absence or presence of Lox and/or AAL-R. The cells were then mixed with CFSE-labeled, GP33-specific $CD8^+$ T cells (Fig. 2D). After 3 d, T cell proliferation rate was analyzed by measuring the decrease of CFSE fluorescence in T cells by flow cytometry. Lox treatment enhanced the ability of DCs to induce T cell expansion (untreated, 10%; Lox/vehicle, 17%, Fig. 2D). Importantly, the addition of AAL-R dramatically promoted T cell proliferation (Lox/AAL-R, 57%, Fig. 2D). Taken together, the sphingosine analogue AAL-R enhances TLR7-mediated DC activation, which is supported by increased expression of MHC-I and costimulatory molecules and inflammatory cytokine synthesis, and enhanced the ability of DCs to induce $CD8^+$ T cell proliferation.

Because GM-CSF enhances DC survival and promotes DC development and differentiation (49), we also investigated the possible effect of the cytokine on the stimulatory activity of AAL-R in DCs. Exogenously supplied GM-CSF increased MHC-I expression on DCs in a dose-dependent manner as expected because of its known function on DC differentiation. AAL-R enhanced Lox-mediated DC maturation independent of GM-CSF supplement (Supplemental Fig. 2). These results suggest that GM-CSF does not alter AAL-R's stimulatory ability on the committed and differentiated DCs.

To confirm the finding obtained with Lox stimulation, another TLR7-specific agonist, imiquimod, was used to stimulate DCs (Fig. 3A). AAL-R evidently increased surface expression of MHC-I (MFI: 78 → 130), B7-1 (MFI: 81 → 111), and CD40 (MFI: 106 → 139) on DCs upon imiquimod treatment. Next, we sought to use TLR7-deficient DCs to rule out the possibility of contamination with other endotoxins that are able to stimulate DCs (Fig. 3B). AAL-R did not affect MHC-I expression on TLR7-deficient DCs upon Lox treatment, whereas its expression on Wt DCs was substantially increased by AAL-R treatment, demonstrating the specificity of TLR7 activation.

Sphingosine and sphingosine analogues can be phosphorylated by sphingosine kinases inside cells. The phosphorylated forms can act intracellularly or be secreted and bind to their cognate S1P receptors to trigger the receptor-mediated signaling pathways (1, 50). We speculated that AAL-R's phosphorylation event is essential for its stimulatory activity in DCs. To determine this, AAL-S, a chiral *S*-enantiomer of AAL-R that is not phosphorylated by sphingosine kinase (17, 18), was used in DC stimulation assays (Fig. 3C). When DCs were treated with AAL-S, the analogue failed to increase MHC-I surface expression on Lox-challenged DCs, whereas AAL-R strongly increased MHC-I expression. The result suggests that phosphorylation of AAL-R is critical for the analogue's stimulation of TLR7-mediated DC maturation.

We next determined the effects of another sphingosine analogue, FTY720, on TLR7-stimulated DC maturation (Fig. 3D). As expected, FTY720 substantially promoted DC phenotypic maturation upon Lox treatment, supported by an increase of the $CD11c^+$/$MHC\text{-}I^{hi}$ population (30% → 43%) and B7-1/2 expression on DCs (data not shown). However, the stimulatory activity of FTY720 was comparatively lower than that of AAL-R ($CD11c^+$/$MHC\text{-}I^{hi}$, 30% → 54%). Presumably, this effect is due to the fact that the phosphorylation rate of AAL-R is greater than that of FTY720 (27). Similar to AAL-R, the stimulatory activity of FTY720 was also dependent on the type of TLRs stimulated (Supplemental Fig. 3). Collectively, these results indicate that sphingosine analogues, not only AAL-R but also FTY720, induce the phenotypic maturation of DCs upon TLR7 stimulation, which involves phosphorylation of the sphingosine analogue.

Previous studies have shown that sphingosine analogues inhibit human monocyte-derived DC development (25, 26). Similar to this observation, AAL-R strongly inhibited the development of

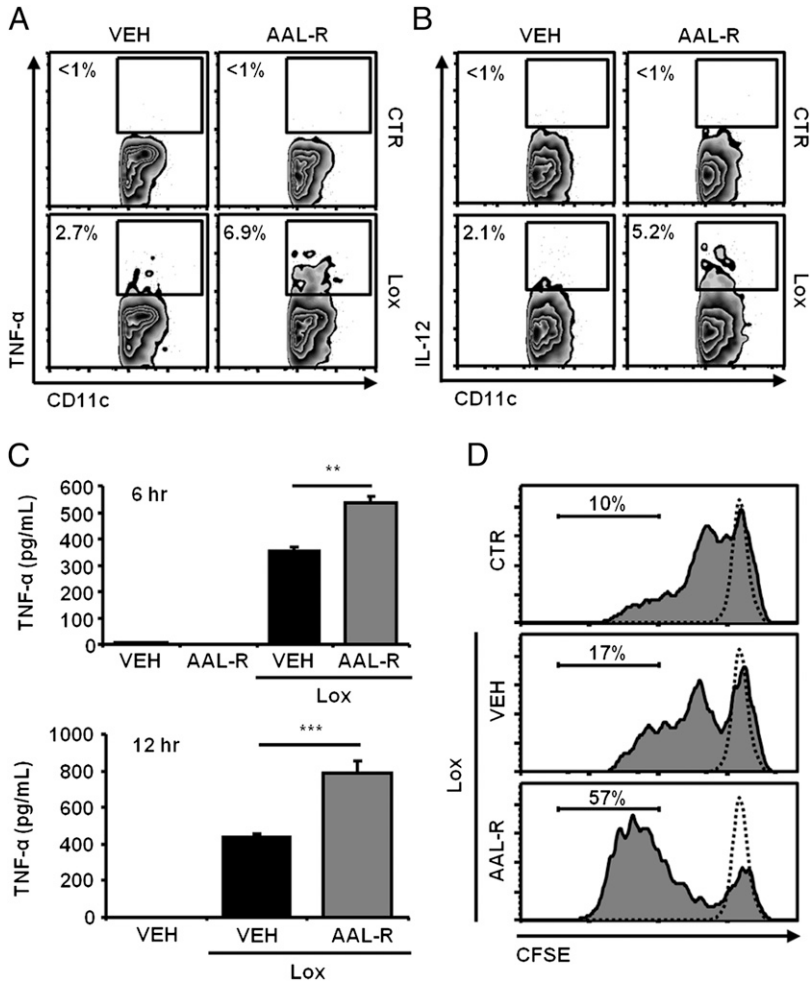

FIGURE 2. AAL-R enhances the function of Lox-treated DCs. (**A** and **B**) DCs were treated with vehicle or AAL-R (1 μM) in the absence or presence of Lox (0.25 mM). At 8 h posttreatment, the intracellular expression of TNF-α (A) and IL-12 (B) in DCs was analyzed by flow cytometry. (**C**) DCs (1×10^6 cells/ml) were treated with vehicle or AAL-R (1 μM) in the presence or absence of Lox (0.5 mM) for 6 (*upper panel*) or 12 (*lower panel*) h. The level of TNF-α in culture supernatants was evaluated by ELISA. Graphs represent the average of TNF-α levels and SEM of data obtained from each condition (n = 3/group). (**D**) DCs were left untreated (CTR) or incubated with Lox (1 mM)/vehicle or Lox (1 mM)/AAL-R (1 μM) in the presence of GP33 peptide for 2 h. Cells were then mixed with CFSE-labeled GP33-specific $CD8^+$ T cells that were isolated from GP33-specific TCR tg mouse. After 3 d, the proliferation of GP33-specific T cells was evaluated by the decrease in CFSE fluorescence in flow cytometric analyses. Histograms show CFSE fluorescence of $CD8^+$ T cells. Dotted lines indicate undivided control cells. The data shown are representative of three or four separate experiments. CTR, control; VEH, vehicle.

$CD11c^+$/MHC-II^+/$CD11b^+$/MHC-I^{high} DCs from BM cells in a GM-CSF–supplemented culture at day 6 (Fig. 4A). Furthermore, AAL-R markedly increased apoptotic populations at day 2 after BM cells were cultured in the presence of GM-CSF (Fig. 4B). These results support the inhibitory activity of AAL-R on the early developmental stage of DCs.

Although AAL-R was shown to induce apoptotic death in splenocytes (17) and BM cells (Fig. 4B), 0.5–1 μM AAL-R did not cause notable apoptotic cell death in DCs (data not shown and Fig. 1E). Apoptosis in Lox-treated DCs was induced only by the treatment with high concentrations of AAL-R such as 4 μM, but not with 2 μM, AAL-R (Fig. 4C). Thus, DCs appear to be relatively resistant to the apoptotic activity of AAL-R. We further determined if the stimulatory activity of AAL-R on DCs is affected by the apoptotic activity of high doses of AAL-R. To this end, DCs were left untreated or treated with 2 or 4 μM AAL-R in the absence or presence of Lox for 8 h (Fig. 4C). AAL-R (2 μM) increased Lox-mediated MHC-I expression on DCs (MFI: 322) compared with vehicle treatment (MFI: 153). However, when DCs were exposed to 4 μM AAL-R, MHC-I expression was dramatically diminished (MFI: 241) compared with 2 μM AAL-R treatment (MFI: 322) (Fig. 4C). A high dose of AAL-R clearly decreased the live cell population (4 μM, annexin V^-/PI^-, 43%) compared with a low dose of AAL-R (2 μM, annexin V^-/PI^-, 65%). However, MHC-I expression on 4 μM AAL-R–treated live cells (annexin V^-/PI^-) still remained as high (MFI: 366) as 2 μM AAL-R–supplied DCs (MFI: 372) (Fig. 4C). These observations indicate that AAL-R has both stimulatory and apoptotic activities in DCs upon TLR7 stimulation.

AAL-R increases DC maturation upon TLR7 stimulation by promoting type I IFN synthesis

The type I IFN family is known as one of the most important factors to enhance DC maturation upon TLR stimulation (29, 36). In this study, we tested if AAL-R treatment affects type I IFN synthesis in DCs upon TLR7 stimulation. IFN-β mRNA expression was detected by qPCR at 6 or 24 h after Lox treatment in the absence or presence of AAL-R (Fig. 5A, 5B). Lox treatment alone induced IFN-β synthesis in DCs at both 6 and 24 h. Notably, additional AAL-R treatment further increased IFN-β synthesis in Lox-challenged DCs 2.6-fold (6 h, Fig. 5A) or 2.9-fold (24 h, Fig. 5B) compared with Lox plus vehicle treatment. Likewise, FTY720 increased IFN-β synthesis ~1.7-fold (Fig. 5B) compared with Lox single treatment. AAL-R–induced augmentation of IFN-β occurred in a concentration-dependent manner (Fig. 5C). The increase of IFN-β by AAL-R upon Lox treatment was not observed when DCs were deficient in TLR7, demonstrating its TLR7 specificity (Fig. 5D). In contrast to AAL-R's stimulatory activity shown with Lox treatment, AAL-R decreased LPS-mediated IFN-β synthesis in DCs 1.7-fold at 6 h compared with LPS single treatment (Fig. 5E), reflecting its inhibitory activity on DC maturation (Supplemental Fig. 1B).

To confirm these results further, IFNAR-deficient DCs were used. Neither Lox nor LPS treatment greatly changed MHC-I expression on IFNAR-deficient DCs (Fig. 5F). Furthermore, AAL-R did not affect MHC-I expression of Lox- or LPS-treated DCs lacking the IFNAR, whereas it evidently changed MHC-I expression on Lox-treated (MFI ± SEM: 48 ± 1 → 83 ± 2) or

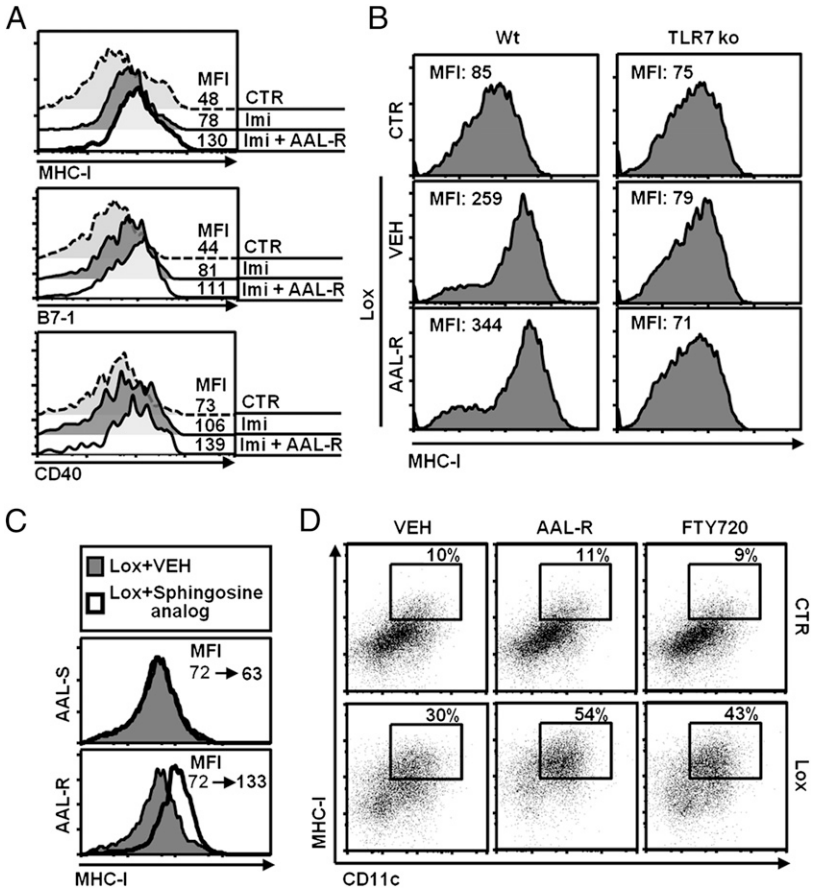

FIGURE 3. Sphingosine analogues increase DC maturation upon TLR7 stimulation. (**A**) DCs were left untreated or treated with vehicle or AAL-R in the presence of imiquimod (Imi; 5 μg/ml) for 1 d. The surface expression of MHC-I, B7-1, and CD40 was analyzed by flow cytometry. (**B**) Wt or TLR7-deficient (TLR7 ko) DCs were left untreated (CTR) or incubated with vehicle or AAL-R (1 μM) in the presence of Lox (1 mM) for 1 d. (**C**) Lox-treated DCs were supplied with AAL-R (1 μM), AAL-S (non-phosphorylatable form of AAL-R, 1 μM, open histogram), or vehicle (shaded histogram). MHC-I surface expression on DCs was analyzed by flow cytometry. MFIs of MHC-I are depicted. (**D**) DCs were treated with vehicle, AAL-R (1 μM), or FTY720 (1 μM) in the absence or presence of Lox (0.5 mM) for 2 d. CD11c/MHC-I expression was assessed by flow cytometry. The percentages of $CD11c^{+}$/$MHC\text{-}I^{high}$ populations are depicted. The experiment was repeated three times with similar results. CTR, control; VEH, vehicle.

LPS-treated (127 ± 3 → 103 ± 3) Wt DCs (Fig. 5F). Similarly, when DCs were defective in IFNAR, neither Lox/vehicle nor Lox/AAL-R treatment enhanced the ability of DCs to induce T cell proliferation (untreated, 3%; Lox/vehicle, 1%; Lox/AAL-R, 1%, Fig. 5G). Finally, we sought to detect any changes in the activation of type I IFN signaling downstream molecules STAT1, STAT2 (42, 51), and ISG15 (52). As a result, AAL-R increased expression of ISG15 and phosphorylated forms of STAT1 and STAT2 (Fig. 5H) upon Lox cotreatment, whereas AAL-R treatment alone did not change their expression (data not shown). However, other signaling molecules of the ERK/MAPK and AKT/PI3K (data not shown) pathways were not affected by AAL-R/Lox cotreatment (Fig. 5H). These observations further support AAL-R's stimulatory activity in TLR7-mediated DC responses through type I IFN signaling. Overall, our results indicate that AAL-R affects TLR-mediated DC maturation by regulating type I IFN production.

AAL-R activates p38 MAPK signaling to increase TLR7-mediated DC maturation

Sphingosine analogues could activate intracellular signaling molecules including AKT, ERK, MEK, and p38 in multiple experimental conditions (53–55). To investigate the intracellular mechanisms of how AAL-R enhances DC responses upon TLR7 stimulation, activation of those molecules was evaluated. Notably, AAL-R strongly increased phosphorylation level of both p38 and its downstream molecule MSK1 in DCs at 2.5 h posttreatment compared with vehicle treatment in the absence or presence of Lox (Fig. 6A). AAL-R–mediated phosphorylation of p38 and MSK1 was detected at 2 or 3 h after Lox treatment, and 4 h later it was decreased (Fig. 6B). However, the phosphorylation levels of ERK, MEK, and AKT were not affected by AAL-R treatment. These observations led us to hypothesize that the p38 MAPK signaling pathway is critical for DC maturation induced by AAL-R upon TLR7 stimulation. To examine this hypothesis, DCs were pretreated with p38 MAPK-specific inhibitor (SB203580) and incubated with AAL-R or vehicle in the presence of Lox. SB203580 blocked the AAL-R–mediated increase of MHC-I (MFI ± SEM: AAL-R, 152 ± 2; AAL-R/SB203580, 110 ± 2) and B7-2 (MFI ± SEM: AAL-R, 107 ± 7; AAL-R/SB203580, 70 ± 2) expression on Lox-treated DCs (Fig. 6C, 6D). In contrast, MEK/MAPK-specific inhibitor (PD98059) failed to block increase of those molecules on DCs (Fig. 6C, 6D). As expected, SB203580 inhibited the phosphorylation of the p38 downstream molecule MSK1 (Fig. 6E), indicating that SB203580 blocked the AAL-R/Lox-induced activation of p38 MAPK signaling pathway. Similarly, the inhibition of p38 MAPK (SB203580), but not MEK (PD98059 or U0126), interfered with AAL-R's stimulatory activity on the production of TNF-α (Fig. 6F), IL-12 (data not shown), and IFN-β (Fig. 6G, 6H). Of note, AAL-R alone in the absence of Lox did not increase the levels of MHC-I and costimulatory molecules and cytokine expression including TNF-α

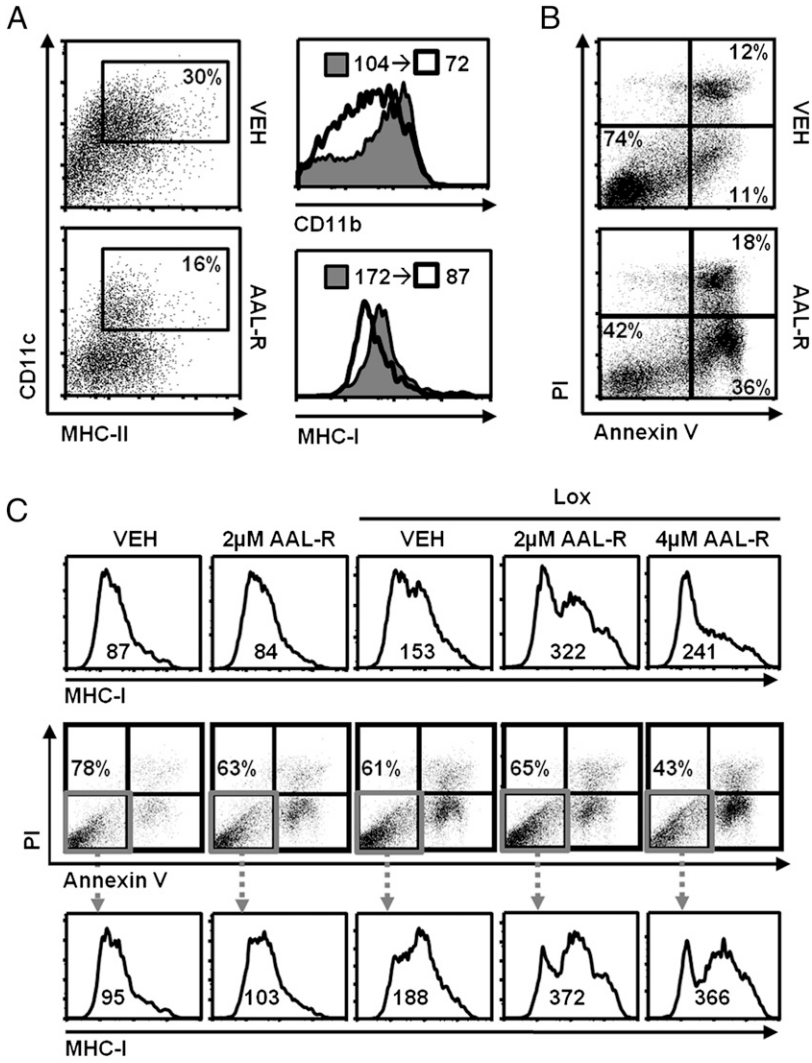

FIGURE 4. The AAL-R regulation of DC differentiation is affected by the status of DCs, which is associated with cellular apoptosis. (**A** and **B**) BM cells were treated with vehicle or AAL-R (1 μM) in the presence of GM-CSF (20 ng/ml). (A) Cells were assessed for the expression of DC surface markers CD11c/MHC-II, CD11b, or MHC-I expression by flow cytometry at day 6. (B) Cells were stained with annexin V–FITC and PI at day 2, then analyzed by flow cytometry. Percentages of annexin V^-/PI^-, annexin V^+/PI^-, and annexin V^+/PI^+ are shown. (**C**) DCs were treated with vehicle or AAL-R (2 or 4 μM) in the absence or presence of Lox for 8 h. Then, cells were washed with PBS twice and additionally cultured for up to 24 h. MHC-I expression on total cells (*upper histograms*) or annexin V^-/PI^- viable cells (*lower histograms*) was analyzed by flow cytometry. The percentages of annexin V^-/PI^- viable cells are depicted in the *middle panel*, and MFIs of MHC-I are shown in the histograms. The data shown are representative of two to four independent experiments. VEH, vehicle.

and IFN-β by DCs even though the p38 MAPK signaling pathway was activated. Therefore, TLR7 stimulation is required for p38-mediated DC activation induced by AAL-R. Collectively, we conclude that AAL-R activates the p38 MAPK pathway to increase type I IFN synthesis and DC maturation upon TLR7 stimulation.

Discussion

In this report, we show that a sphingosine analogue enhances DC responses upon TLR7 stimulation, and its cellular mechanism involves the p38 MAPK and type I IFN signaling pathways. Previously, sphingosine analogues displayed immunosuppressive activity and therefore have been evaluated to alleviate excessive or aberrant immune responses that are associated with immune disorders. The sphingosine analogues were reported to impair DC activation and regulate lymphocyte trafficking (22, 23). For example, FTY720 suppressed DC functions, which resulted in reduced inflammation in an experimental asthma model (8). Similarly, human monocyte-derived DCs generated in the presence of FTY720 displayed an impaired immunostimulatory capacity (26). In addition, upon influenza virus infection, the locally delivered sphingosine analogue AAL-R acted on pulmonary DCs to inhibit influenza virus-specific $CD8^+$ T cell responses (13). Consistent with these inhibitory activities in DCs, AAL-R hindered the generation of DCs from BM progenitor cells in a GM-CSF–supplemented culture system (Fig. 4A). Surprisingly, we have found that AAL-R differently regulates TLR-mediated DC maturation depending on the types of PAMPs. AAL-R treatment increased TLR2-, TLR7-, or TLR9-mediated DC maturation, whereas it suppressed TLR3- or TLR4-mediated DC maturation (Supplemental Fig. 1A). Given that TLR2, TLR7, and TLR9 signaling depend on the MyD88 pathway (30, 45), AAL-R's stimulatory activity may require the activation of the MyD88 signaling pathway triggered by TLR2, TLR7, or TLR9 ligation. However, TLR3 signaling is dependent on the TIR-domain–containing adapter-inducing IFN-β (TRIF) pathway, and TLR4 signaling is reliant on both MyD88 and TRIF signal pathways (30, 45). Therefore, it is conceivable that AAL-R's suppressive function in DCs may necessitate activation of the TRIF signaling upon

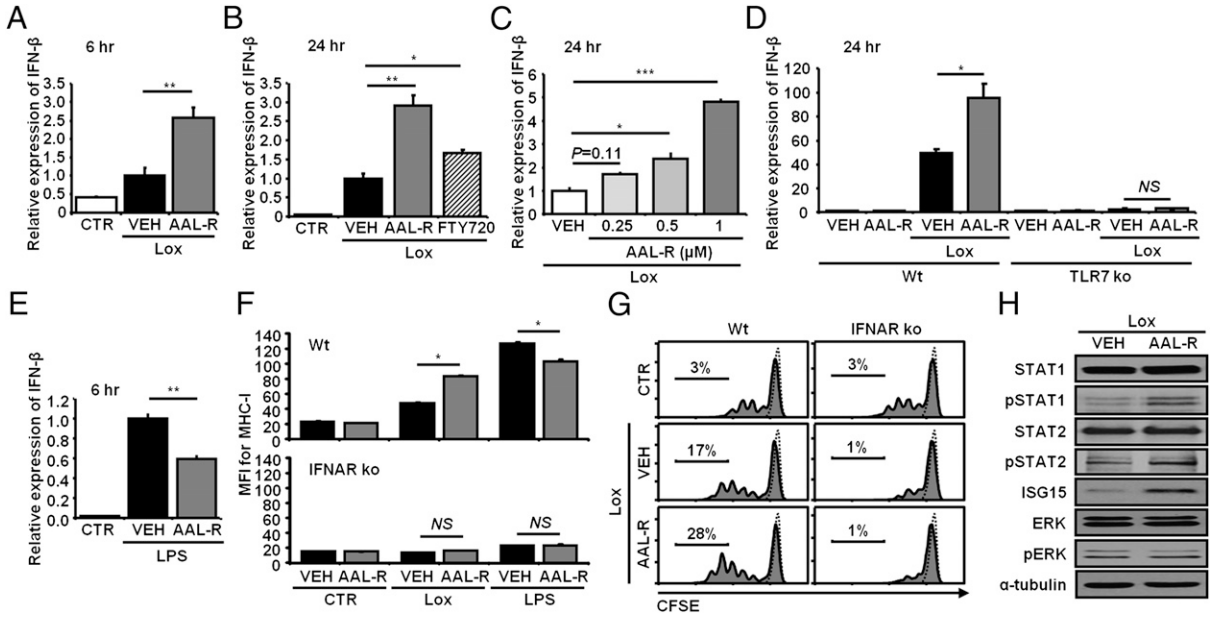

FIGURE 5. AAL-R increases DC maturation upon TLR7 stimulation by promoting type I IFN production. Wt (**A**–**E**) or TLR7-deficient (D) DCs were left untreated (CTR) or treated with Lox (1 mM) (A–D) or LPS (200 ng/ml) (E) in the absence or presence of AAL-R or FTY720 (B) for 6 h (A, E) or 24 h (B–D). The qPCR was performed to detect the relative mRNA level of IFN-β. Graphs represent the average of relative IFN-β expression and SEM of data obtained from each condition ($n = 3$/group). (**F**) Wt or IFNAR-deficient (IFNAR ko) DCs were left untreated (CTR) or treated with Lox (1 mM) or LPS (200 ng/ml); these cells were supplied with AAL-R (1 μM) for 1 d. The MFIs of MHC-I expression on DCs were determined by flow cytometry. Graphs show the average of MFI and SEM ($n = 3$/group). (**G**) Wt or IFNAR ko DCs were left untreated (CTR) or incubated with Lox (1 mM)/vehicle or Lox (1 mM)/AAL-R (1 μM) in the presence of GP33 peptide for 2 h. Cells were then mixed with CFSE-labeled GP33-specific $CD8^+$ T cells. After 3 d, the proliferation of GP33-specific T cells was evaluated by flow cytometric analyses. Histograms show CFSE fluorescence of $CD8^+$ T cells. Dotted lines indicate undivided control cells. (**H**) DCs were treated with vehicle or AAL-R (1 μM) in the presence of Lox (1 mM) for 2 d. Cell lysates were used for Western blot analysis to detect STAT1, p-STAT1, STAT2, p-STAT2, ISG15, ERK, p-ERK, and α-tubulin. The data shown are representative of three to five independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. CTR, control; VEH, vehicle.

TLR3 or TLR4 ligation on DCs, and this could be dominant over MyD88-mediated stimulatory action when both signaling pathways are activated by TLR4 ligation. Therefore, our data demonstrate that the notable stimulatory or suppressive activity of the sphingosine analogue AAL-R is associated with PAMPs-specific DC responses.

Importantly, AAL-R's stimulatory activity was mediated through type I IFN signaling. This is strongly supported by our results. First, AAL-R increased IFN-β mRNA synthesis upon TLR7 engagement (Fig. 5A–C). Second, activation of the downstream signaling molecules STAT1/2 and enhanced expression of a type I IFN-inducible gene, ISG15, were observed in AAL-R–conditioned DCs (Fig. 5H). Third, the stimulatory activity of AAL-R was nullified when IFNAR-mediated signaling was lacking in DCs by genetic deletion of the receptor upon Lox treatment (Fig. 5F, 5G). Thus, our data imply that sphingosine signaling pathways interact with the TLR7 signaling pathway to enhance the production of type I IFNs.

We have also shown that the p38 MAPK signaling activated by AAL-R is important to increase type I IFN synthesis and DC maturation upon TLR7 stimulation. Although sphingosine signaling could trigger diverse intracellular signaling pathways including AKT/PI3K, ERK/MAPK, and p38 MAPK signaling pathways (53–55), our data indicate that the sphingosine analogue AAL-R specifically activates the p38 MAPK signaling pathway, but not the AKT/PI3K and ERK/MAPK pathways in DCs. Importantly, the p38 MAPK signaling pathway is known to play a critical role in the expression of type I IFNs (56, 57). In support of this, blockade of p38 MAPK activation impaired the AAL-R–mediated expression of type I IFN (Fig. 6G). Because AAL-R–mediated DC activation is highly dependent on type I IFN signaling upon TLR7 stimulation (Fig. 5F, 5G), p38 MAPK signaling pathway activated by AAL-R could be a main mechanism to enhance DC responses via increased type I IFN synthesis.

Because AAL-R/FTY720 could activate S1PR1 and S1PR 3–5 (17, 18), we determined the effect of agonists for S1PR1, S1PR3, and S1PR4 on DC maturation. However, these agonists did not increase DC maturation upon TLR7 stimulation in the experimental condition (data not shown). Therefore, it is possible that this newly found stimulatory function of AAL-R is mediated by intracellular activity of AAL-R after its phosphorylation by SphK2 or S1PR5-mediated signaling. Although both SphK1 and SphK2 generate S1P from sphingosine (16), these two enzymes are localized in different sites (SphK1, cytoplasm; SphK2, nucleus) in the cells (58), often display opposed activities on cellular processes (59, 60), and the regulatory mechanism is not clearly defined. This suggests that there are factors such as S1P localization that affect its functions. We found that the phosphorylation of AAL-R is critical for the stimulatory activity on DC maturation. Although any analogues may display off-target effects especially when used at very high concentration, the off-target of AAL-R/FTY720 has not been definitely identified to our knowledge. Further, the AAL-R activity occurred in a dose-dependent manner, and the concentration of AAL-R (0.25–1 μM) we used is not higher than the doses (0.5–10 μM) that have been used by other investigators (13, 17). Thus, it is likely that AAL-R is phosphorylated by SphK2 and may act on as-yet unidentified target in the S1P signaling that could be linked with p38 and type I IFN signal pathways to stimulate DCs upon TLR7 ligation. The detailed molecular signaling mechanisms remain to be determined.

We have shown that treatment with a high dose of AAL-R induces apoptotic death of DCs, which affects DC maturation (Fig. 4C), although DCs were more resistant to AAL-R–induced

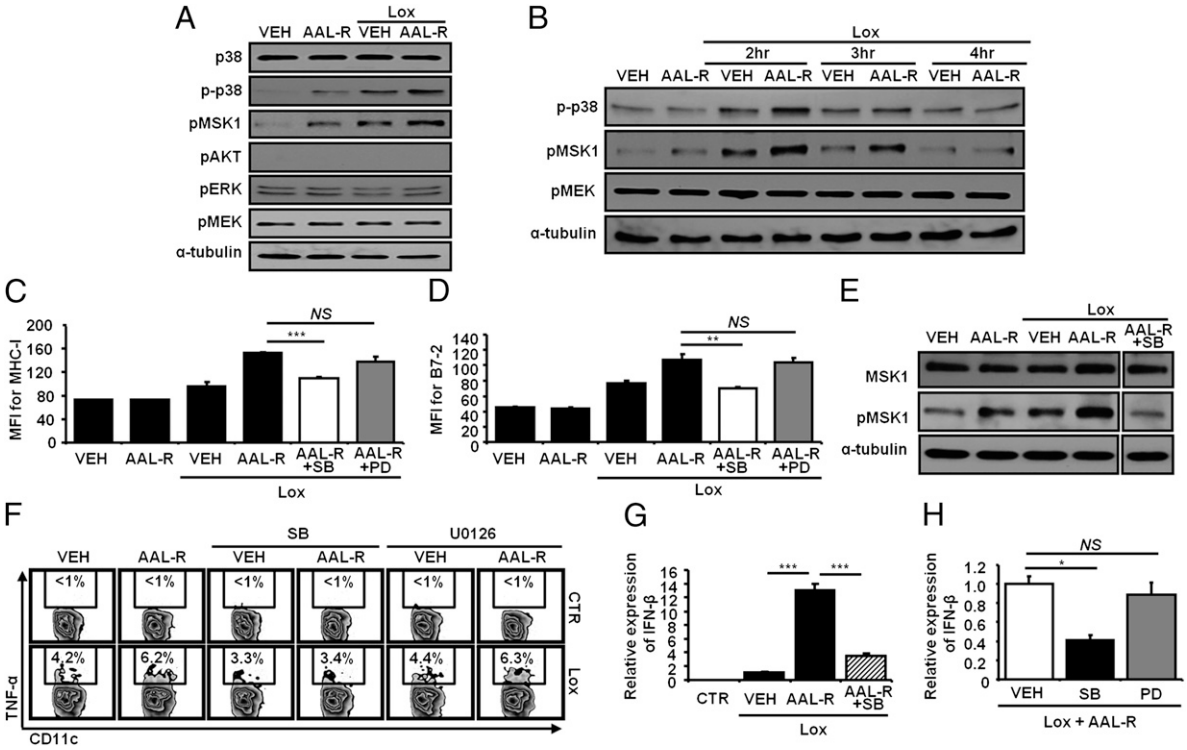

FIGURE 6. AAL-R increases DC responses via activation of p38 signaling pathway. (**A** and **B**) DCs were treated with vehicle or AAL-R (1 μM) in the absence or presence of Lox (0.5 mM) for 2.5 h (A) and 2, 3, or 4 h (B). Cell lysates were used for Western blot analysis to detect p38, p-p38, p-MSK1, p-AKT, p-ERK, p-MEK, and α-tubulin. (**C** and **D**) DCs were pretreated with an inhibitor blocking pathways for p38 (SB203580, 10 μM) or MEK/MAPK (PD98059, 10 μM) for 1 h. Then, cells were treated with vehicle or AAL-R (1 μM) in the absence or presence of Lox (0.5 mM). At 24 h posttreatment, the surface expression of MHC-I (C) and B7-2 (D) was analyzed by flow cytometry. Graphs show the average of MFI and SEM (n = 3/group). (**E**) DCs were treated as in (C) and (D) for 3 h. Then, cells were harvested to detect MSK1, p-MSK1, and α-tubulin by Western blot analysis. (**F**) DCs were pretreated with SB203580 (10 μM) or MEK/MAPK inhibitor U0126 (10 μM) for 1 h, and then cells were incubated with vehicle or AAL-R for an additional 8 h in the presence or absence of Lox. Intracellular cytokine staining was performed to detect TNF-α in DCs, and its intracellular level was analyzed by flow cytometry. (**G** and **H**) DCs were treated as in (C) and (D) for 8 h. Then, cells were harvested to detect the relative mRNA expression level of IFN-β by qPCR. Graphs represent the average of relative IFN-β expression and SEM of data obtained from each condition (n = 5/group). The experiments were repeated two to four times with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. PD, PD98059; SB, SB203580; VEH, vehicle.

cell death (Fig. 4C) compared with BM cells (Fig. 4B) and splenocytes (17). We have also demonstrated that AAL-R differentially regulates TLR-mediated DC responses (Supplemental Fig. 1A). Therefore, AAL-R's modulatory activity in DCs seems to be affected by diverse factors including DC differentiation status, type of stimulus, and strength of AAL-R–mediated signaling.

Recently, FTY720 treatment was approved by the Food and Drug Administration for the treatment of multiple sclerosis as an orally administered drug (12). However, patients could be exposed to diverse pathogens, which may affect the drug's efficacy. Indeed, several patients treated with FTY720 died upon viral infections (61), and how FTY720 affected host immune responses to the infections in those patients remains unknown. Therefore, it is imperative to understand how FTY720 regulates host immune responses to infections in a pathogen-specific manner. Further mechanistic studies to separate the immunostimulatory from immunosuppressive properties of FTY720 will provide important data that would be therapeutically applicable.

The therapeutic potential of sphingosine analogues upon influenza virus infection was evaluated in recent studies (13, 14). The local administration of sphingosine analogue AAL-R in mice dampened the cytokine responses and DC activation caused by influenza virus infection, representing AAL-R's inhibitory activity. Although influenza virus has ssRNA genomes and thus host immune cells may recognize the viruses with host PRRs including TLR7, we have shown that AAL-R could increase DC responses upon TLR7 stimulation. There are two possibilities to explain this difference. First, influenza virus infection induces cell death to produce progeny viruses. It is possible that AAL-R's apoptotic or immunosuppressive potential cooperates with influenza viral cytopathogenic activity leading to the repressed immune responses. Second, influenza virus may activate other PAMPs in addition to TLR7. The influenza virus-specific PAMPs may bind to the cognate cellular receptor and trigger its downstream signaling, but this process may be negatively regulated by AAL-R, leading to AAL-R–mediated suppression of DC responses to the infection.

Owing to the extraordinary capacity of DCs to act as natural adjuvants, the potential of Ag-mounted DCs for the treatment of diseases has been confirmed in multiple experimental models (28, 62, 63). We have shown that AAL-R differentially regulates DC responses depending on the type of TLR activation. Also, our data revealed the stimulatory activity of sphingosine analogue AAL-R in DC responses after TLR7 stimulation. The sphingosine analogues' immunostimulatory action mode could be applicable to the development of novel DC-mediated immunotherapeutic strategies to remedy multiple diseases caused by diverse pathogenic microbes.

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Disclosures

The authors have no financial conflicts of interest.

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