

MicroRNA-9 regulates steroid-resistant airway hyperresponsiveness by reducing protein phosphatase 2A activity

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Background: Steroid-resistant asthma is a major clinical problem that is linked to activation of innate immune cells. Levels of IFN- γ and LPS are often increased in these patients. Cooperative signaling between IFN- γ /LPS induces macrophage-dependent steroid-resistant airway hyperresponsiveness (AHR) in mouse models. MicroRNAs (miRs) are small noncoding RNAs that regulate the function of innate immune cells by controlling mRNA stability and translation. Their role in regulating glucocorticoid responsiveness and AHR remains unexplored. **Objective:** IFN- γ and LPS synergistically increase the expression of miR-9 in macrophages and lung tissue, suggesting a role in the mechanisms of steroid resistance. Here we demonstrate the role of miR-9 in IFN- γ /LPS-induced inhibition of dexamethasone (DEX) signaling in macrophages and in induction of steroid-resistant AHR. **Methods:** MiRNA-9 expression was assessed by means of quantitative RT-PCR. Putative miR-9 targets were determined *in silico* and confirmed in luciferase reporter assays. miR-9 function was inhibited with sequence-specific antagomirs. The efficacy of DEX was assessed by quantifying glucocorticoid receptor (GR) cellular localization, protein phosphatase 2A (PP2A) activity, and AHR.

Results: Exposure of pulmonary macrophages to IFN- γ /LPS synergistically induced miR-9 expression; reduced levels of its target transcript, protein phosphatase 2 regulatory subunit B (B56) δ isoform; attenuated PP2A activity; and inhibited DEX-induced GR nuclear translocation. Inhibition of miR-9 increased both PP2A activity and GR nuclear translocation in macrophages and restored steroid sensitivity in multiple models of steroid-resistant AHR. Pharmacologic activation of PP2A restored DEX efficacy and inhibited AHR. MiR-9 expression was increased in sputum of patients with neutrophilic but not those with eosinophilic asthma.

Conclusion: MiR-9 regulates GR signaling and steroid-resistant AHR. Targeting miR-9 function might be a novel approach for the treatment of steroid-resistant asthma. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: MicroRNA, pulmonary macrophages, innate immunity, protein phosphatase 2A, steroid-resistant airway hyperresponsiveness

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Asthma is a heterogeneous disorder divided into a number of distinct subsets (phenotypes) based on airway inflammatory cell profiles.¹⁻⁴ Glucocorticoids are a frontline anti-inflammatory treatment for disease management; however, some patients respond poorly to therapy, even at high doses.^{5,6} Steroid resistance occurs predominantly in patients with severe and neutrophilic asthma in whom innate immune cells (eg, neutrophils and macrophages rather than eosinophils) are activated and thought to contribute to pathogenesis.⁷⁻¹⁰ An improved understanding of the mechanisms underlying steroid resistance and alternative approaches to overcome poor treatment responsiveness are urgently required.

Numerous lines of evidence suggest a link between innate immune activation and steroid-resistant asthma. Activated macrophages and neutrophils are prominent features in the inflammation linked to steroid-resistant disease.^{4,10} In patients early-life infections are linked to increased asthma susceptibility, exacerbations, and a decrease in lung function.^{8,11-13} Recently, we demonstrated novel synergistic signaling between IFN- γ and LPS (2 factors linked to the pathogenesis of steroid-resistant asthma), which induces macrophage-dependent steroid-resistant airway hyperresponsiveness (AHR) in a mouse model.^{9,14} We also demonstrated that macrophages and IFN- γ induce persistent AHR in an allergen-induced model of steroid-resistant asthma.¹⁵ Furthermore, we demonstrated that microRNAs (miRNAs) play a pivotal role in allergic inflammation in mouse asthma models^{16,17}; however, the role of miRNAs in steroid resistance remains unknown.

Abbreviations used

AAL(S):	(S)-2-Amino-4-(4-[heptyloxy]phenyl)-2-methylbutan-1-ol
AHR:	Airway hyperresponsiveness
ant-9:	Antagomir-9
DEX:	Dexamethasone
GR:	Glucocorticoid receptor
JNK:	c-Jun N-terminal kinase
miR:	MicroRNA
OVA:	Ovalbumin
PP2A:	Protein phosphatase 2A
PPP2R2A:	Protein phosphatase 2 regulatory subunit B (B56) α isoform
PPP2R5D:	Protein phosphatase 2 regulatory subunit B (B56) δ isoform
UTR:	Untranslated region

MiRNAs are short (19–25nt) noncoding RNAs that regulate gene expression, inhibiting translation or destabilizing target mRNAs.^{18,19} MiRNAs bind target mRNA 3' untranslated regions (UTRs) with partial complementarity, and therefore a single miRNA can target multiple mRNAs. This allows miRNAs to fine tune specific target expression or act broadly on signaling pathways controlling immune cell function.^{20–24} Because miRNAs play important roles in maintaining cellular function, dysregulation can predispose to disease (eg, cancer and heart disease). However, the functional roles of miRNAs in patients with inflammatory and immune disorders are just beginning to be explored.^{25–31} Already, a subset of miRNAs has been identified as potential therapeutic targets in asthmatic patients.^{16,17,32–34} MiR-126 and miR-21 have been implicated in mouse eosinophilic asthma models. Furthermore, we showed that targeted inhibition of miR-126 reduces clinical symptoms by inhibiting T_H2 inflammatory responses.¹⁷ Other miRNAs (eg, miR-18a, miR-128b, and miR-221) have also been implicated in the regulation of glucocorticoid function, modulating glucocorticoid receptor (GR) expression.^{35,36} However, the role of miRNAs in the regulation of steroid-resistant asthma pathways remains unclear.

In the current study we demonstrate that miR-9 expression is increased in pulmonary macrophages and lung samples in our IFN- γ /LPS-induced mouse model of steroid-resistant AHR and in sputum samples from patients with neutrophilic asthma. MiR-9 expression decreased protein phosphatase 2A (PP2A) activity and dexamethasone (DEX)-induced GR nuclear translocation through targeting of protein phosphatase 2 regulatory subunit B (B56) δ isoform (PPP2R5D), a key regulatory subunit of PP2A. Specific inhibition of miR-9 enhanced PP2A activity and restored DEX-induced GR nuclear translocation. Furthermore, *in vivo* miR-9 inhibition or PP2A activation with (S)-2-amino-4-(4-[heptyloxy]phenyl)-2-methylbutan-1-ol (AAL[S]) restored steroid efficacy, reducing AHR when administered with DEX. MiR-9 inhibition also restored steroid efficacy in mouse models of infection-induced AHR exacerbation, suggesting miR-9 as a novel target for treatment of steroid-resistant inflammation and AHR.

METHODS**Mice**

Wild-type specific pathogen-free BALB/c mice (6–8 weeks old) were obtained from the University of Newcastle. All experiments were performed with approval from the local animal ethics committee.

Isolation and stimulation of pulmonary macrophages

Pulmonary macrophages were isolated from lungs, as previously described.³⁷ Lung tissue was minced, and cell suspensions were prepared, separated by means of density gradient centrifugation (Histopaque-1083; Sigma-Aldrich, St Louis, Mo), and plated at 6×10^6 cells/mL in Dulbecco modified Eagle medium containing 20% FCS. After 3 hours, 95% of adherent cells were macrophages, as confirmed by means of flow cytometry, which were cultured overnight and stimulated as indicated. Detection of GR localization by means of immunofluorescence and assessment of target protein expression by means of Western blotting are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Collection of induced sputum samples

Collection of induced sputum samples is described in detail in the [Methods](#) section in this article's Online Repository.

Induction of IFN- γ /LPS-induced steroid-resistant AHR

Mice were anesthetized (100 μ L of Alfaxan solution [1:4; Jurox, Malvern, United Kingdom] in PBS administered intravenously) and intubated with a 22-gauge catheter.¹⁴ Optimized doses of murine IFN- γ (1.5 μ g per mouse; PeproTech, Rocky Hills, NJ), LPS (50 ng per mouse, Sigma-Aldrich), or IFN- γ plus LPS (IFN- γ /LPS; 1.5 μ g plus 50 ng per mouse) in 50 μ L of vehicle (saline) were instilled intratracheally. After 12 hours (peak of steroid-resistant AHR), AHR was measured, and lung samples were collected (see the [Methods](#) section in this article's Online Repository). Where specified, mice were treated with DEX (1 mg/kg administered intraperitoneally twice per week), the nonphosphorylatable FTY720 analog AAL(S) (10 μ g per mouse administered intranasally daily), or both.

Induction of allergic airway inflammation and persistent steroid-resistant AHR

Mice were sensitized with ovalbumin (OVA; 50 mg administered intraperitoneally; fraction V, Sigma-Aldrich) and Alhydrogel (1 mg; Reheis, Berkeley Heights, NJ) in 200 μ L of 0.9% sterile saline. Nonsensitized control mice were injected with Alhydrogel alone. On days 13 to 16, mice were aerosol challenged with OVA (10 mg/mL in 0.9% saline) for 20 minutes. Mice were also exposed to LPS (50 ng per mouse administered intranasally; Sigma-Aldrich [to mimic infection]) with or without DEX (1 mg/kg administered intraperitoneally, Sigma-Aldrich). On day 17 or 1 week after the last OVA challenge, AHR was assessed and inflammatory infiltrates were quantified, as previously described¹⁴ and shown in the [Methods](#) section in this article's Online Repository.

Antagomir treatment

The miR-9 sequence was obtained from miRBase (Wellcome Trust, Sanger Institute, Cambridge, United Kingdom; <http://microrna.sanger.ac.uk/sequences/>). Antagomirs for miR-9 and the scrambled control (nonspecific RNA VIII, blasted against the mouse genome) were ordered from Dharmacon (Lafayette, Colo). The sequence of ant-miR-9 was (5'–3') as follows: 5'mT.*mC.*mA.mT.mA.mC.mA.mG.mC.mT.mA.mG.mA.mT.mA.

mA.mC.mC.mA.*mA.*mA.*mG.mA.*3'-Chol, where *m* indicated 2-OMe-modified phosphoramidites, * indicates phosphorothioate linkages, and -Chol indicates hydroxypropylol-linked cholesterol. Antagomir-9 (ant-9; miR-9 sequence-specific antagomir) or scrambled antagomir control (50 μ g per mouse) were administered intratracheally 3 times, as indicated in the respective figure. Quantitative assessment of

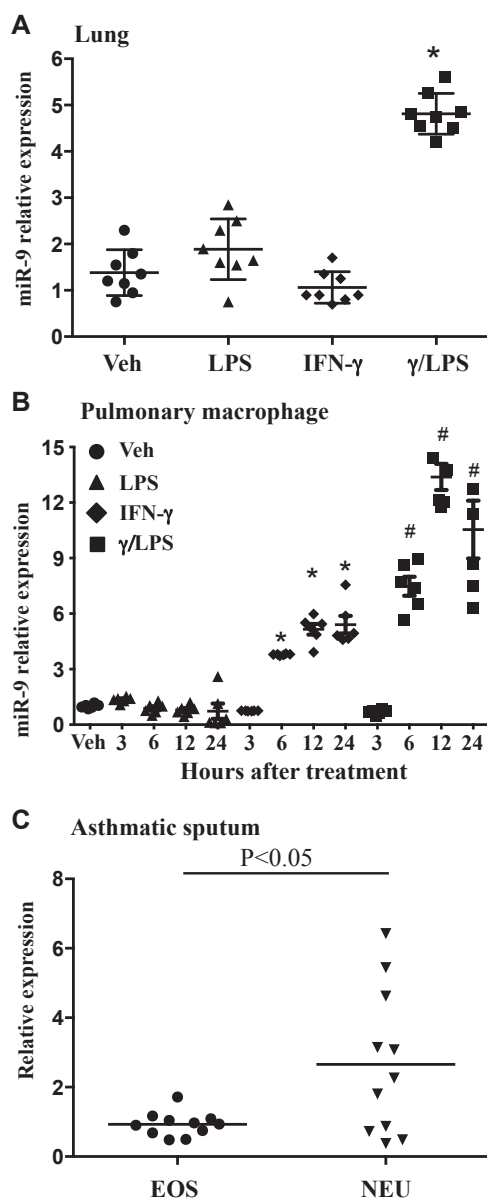


FIG 1. MiR-9 expression is induced by IFN- γ /LPS stimulation. **A**, MiR-9 expression levels in lung samples from mice 12 hours after exposure to LPS, IFN- γ , or IFN- γ /LPS. Data are means $\pm$ SEMs (n = 8 mice per group). **B**, Pulmonary macrophages stimulated with LPS, IFN- γ , or IFN- γ /LPS (γ /LPS) for 3, 6, 12, or 24 hours. Each dot represents the mean of an independent experiment performed in duplicate. **C**, Induced sputum samples from asthmatic patients. EOS, Eosinophilic; NEU, neutrophilic; Veh, vehicle control. Values are means $\pm$ SEMs. * P < .05 versus vehicle control. # P < .05 versus vehicle control and LPS or IFN- γ treatment at respective time points.

miRNAs and mRNA expression is described in the [Methods](#) section in this article's Online Repository.

MiRNA target prediction and luciferase reporter assay

MiR-9 mRNA targets were predicted by using TargetScan (Whitehead Institute For Biomedical Research, Cambridge, Mass).³⁸ Predicted targets were limited to genes with known involvement in glucocorticoid-signaling pathways. Luciferase reporter assays are detailed in the [Methods](#) section in this article's Online Repository.

PP2A activity assay

PP2A activity was assessed by using the Active PP2A DuoSet IC Activity assay kit (R&D Systems, Minneapolis, Minn), according to the manufacturer's specifications.

Data analysis

Two-way ANOVA was used to identify differences in lung function, and Student t tests were used for all other comparisons. The Mann-Whitney U test was used to compare differences in miR-9 expression between samples from patients with neutrophilic asthma and those from patients with eosinophilic asthma. Values are presented as means $\pm$ SEMs for 8 to 12 samples per group, and differences were considered significant at a P value of less than .05.

RESULTS

MiR-9 expression is increased under steroid-resistant AHR conditions

To explore roles for miRNAs in patients with steroid-resistant AHR, we performed microarrays on lung samples from IFN- γ /LPS-treated mice (see the [Methods](#) in this article's Online Repository) to identify miRNAs with altered expression (see [Table E1](#) in this article's Online Repository at www.jacionline.org). Among these miRNAs, miR-9 levels were markedly increased after administration of IFN- γ /LPS compared with those after IFN- γ or LPS alone, which was further confirmed by using quantitative PCR ([Fig 1, A](#)). Furthermore, this pattern linked miR-9 to steroid-resistant AHR because airway hyperreactivity was only induced by combined IFN- γ /LPS treatment.¹⁴ On the basis of the critical role of macrophages in steroid-resistant AHR, we assessed miR-9 expression in pulmonary macrophages treated with LPS, IFN- γ , or IFN- γ /LPS for 3, 6, 12, and 24 hours. Quantitative PCR demonstrated a modest increase in miR-9 expression after exposure to IFN- γ but not LPS ([Fig 1, B](#)). Furthermore, miR-9 expression was synergistically upregulated by IFN- γ /LPS treatment, peaking at 12 hours ([Fig 1, B](#)). Interestingly, miR-9 expression was also increased in sputum samples from patients with neutrophilic asthma compared with those from patients with eosinophilic asthma ([Fig 1, C](#)). These findings demonstrate that miR-9 is induced in pulmonary macrophages and lung tissue under conditions that induce macrophage-dependent steroid-resistant AHR, as well as in a subset of asthmatic patients who are often resistant to steroid therapy.

Inhibition of miR-9 restores steroid sensitivity, reducing AHR induced by IFN- γ /LPS

To assess the functional role of miR-9 in patients with steroid-resistant AHR, we inhibited miR-9 through intratracheal administration of ant-9 before IFN- γ /LPS exposure. Ant-9 administration effectively blocked miR-9 induction in the lung ([Fig 2, A and C](#)) but alone had no effect on AHR induced by IFN- γ /LPS ([Fig 2, B](#)). As we have previously described, IFN- γ /LPS induces steroid-resistant AHR, which is not inhibited by DEX treatment ([Fig 2, D](#)).⁹ However, inhibition of miR-9 in conjunction with DEX attenuated AHR ([Fig 2, D](#)). Although ant-9 plus DEX treatment inhibited AHR, ant-9 administration had no effect on inflammatory cell lung infiltration (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). These findings

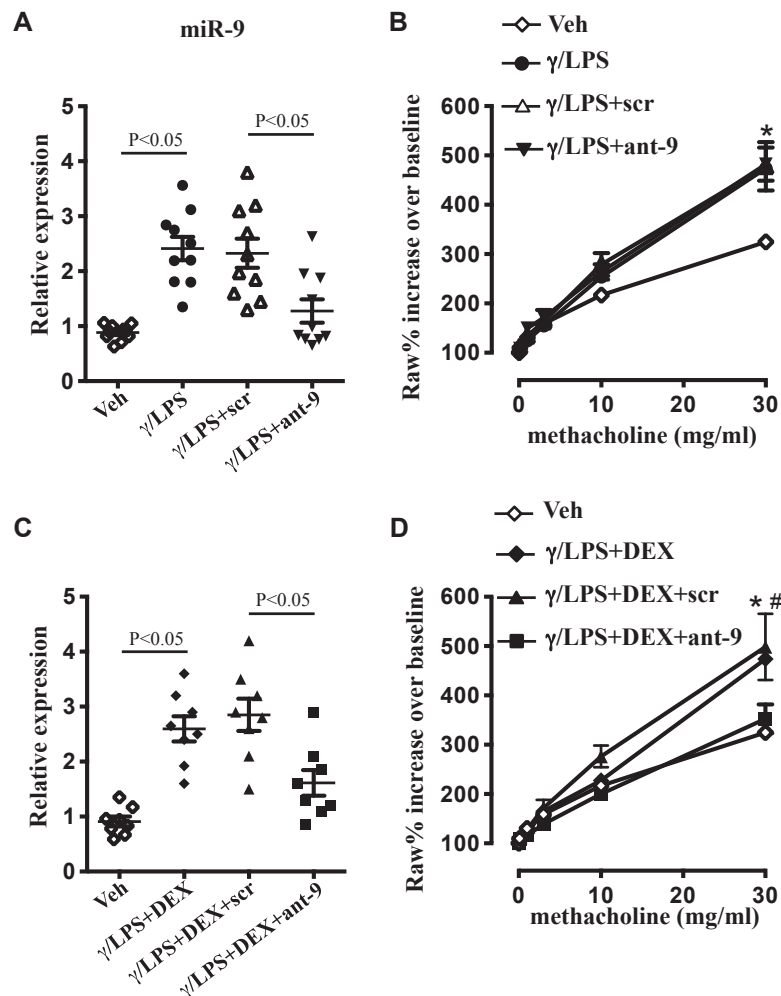


FIG 2. Inhibition of miR-9 restores DEX sensitivity, attenuating AHR induction by IFN- γ /LPS (γ /LPS). Mice were administered ant-9 or scrambled antagomir (*scr*), DEX, or both 12 hours before IFN- γ /LPS exposure. Twelve hours later, lung function was assessed, and lung tissues were isolated. MiR-9 levels in lung normalized to sno-202 (A), lung function measurements after exposure to IFN- γ /LPS and ant-9 or scr (B), miR-9 levels (C), and lung function measurements of mice pretreated with DEX and ant-9 or scr (D) are shown. Values are means $\pm$ SEMs (n = 8 to 10 mice per group). Veh, Vehicle control. *P < .05 versus vehicle control. #P < .05, γ /LPS+DEX+scr versus γ /LPS+DEX+ant-9.

indicate that ant-9 exposure alone has no effect on AHR, despite reducing miR-9 levels; however, ant-9 treatment restores steroid sensitivity.

MiR-9 expression inhibits PP2A activity by targeting PPP2R5D

To clarify the mechanism underlying miR-9 inhibition of steroid sensitivity, we performed *in silico* target prediction for proposed miR-9 target molecules (see Table E2 in this article's Online Repository at www.jacionline.org) potentially involved in defective GR signaling observed in patients with steroid-resistant disorders.^{6,39-42} This assessment highlighted 2 PP2A regulatory subunits, PPP2R5D and protein phosphatase 2 regulatory subunit B (B56) α isoform (PPP2R2A). Predicted miR-9 target sites are conserved within the 3'UTR of PPP2R5D and PPP2R2A, matching the miR-9 seed sequence at position 398-404 and position 1361-1367, respectively (Fig 3, A, and see Table E2). To verify miR-9 targeting of these transcripts, we

cloned the 3'UTR regions of each gene into dual-fluorescence luciferase reporter constructs. Cotransfection of luciferase constructs with miR-9 mimic resulted in a dose-dependent decrease in luciferase activity compared with that seen in control mimic. Furthermore, deletion of the miR-9 recognition site at the 3'UTR regions of each gene led to no reduction in luciferase activity (Fig 3, B). These data demonstrate that miR-9 directly targets PPP2R5D and PPP2R2A transcripts.

To determine whether PPP2R5D or PPP2R2A are regulated by miR-9 in our model, we assessed mRNA expression in IFN- γ /LPS-stimulated macrophages with or without ant-9. Quantitative PCR confirmed efficient knockdown miR-9 by ant-9 (Fig 4, A). PPP2R5D expression was reduced after IFN- γ /LPS stimulation (Fig 4, B-D) and restored by ant-9 treatment (Fig 4, B-D), indicating that PPP2R5D is downregulated by miR-9 in pulmonary macrophages. Interestingly, miR-9 inhibition had limited effects on PPP2R2A, with slight decreases in PPP2R2A variant 1 expression after ant-9 treatment (see Fig E2 in this article's Online Repository at www.jacionline.org).

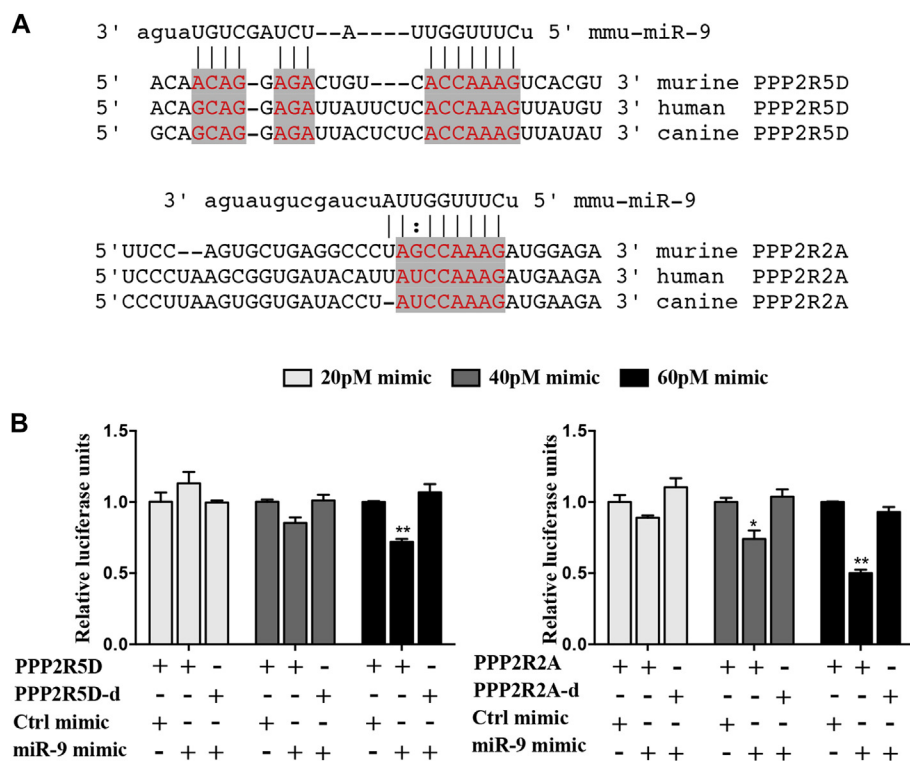


FIG 3. Targeting of PPP2R5D and PPP2R2A mRNA by miR-9. **A**, Conserved miR-9-binding sites (highlighted) in the 3'UTR of PPP2R5D and PPP2R2A mRNAs. **B**, Luciferase activity in lysates of HEK293 cells transfected with constructs encoding the 3'UTR regions of PPP2R5D or PPP2R2A and miR-9 mimic or scrambled control at indicated concentrations. Values are means $\pm$ SEMs of 3 independent experiments performed in triplicate. * $P < .05$ and ** $P < .005$.

Because PPP2R5D levels affect PP2A catalytic activity,⁴³⁻⁴⁶ we hypothesized that increased miR-9 expression might reduce overall PP2A catalytic activity. PP2A activity was reduced after IFN- γ /LPS treatment, and ant-9 significantly recovered this activity compared with scrambled control in macrophages (Fig 4, E). Assessment of PP2A activity in IFN- γ /LPS-stimulated lung samples revealed similar findings, with increased miR-9 expression and reduced PP2A enzyme activity (Fig 4, F). Western blots also demonstrated increased c-Jun N-terminal kinase (JNK) phosphorylation 12 hours after IFN- γ /LPS administration to pulmonary macrophages, which was inhibited by ant-9 pretreatment (Fig 4, G and H). Taken together, these findings suggest that increased miR-9 expression reduced expression of its target, PPP2R5D, leading to reduced PP2A activity, increased JNK phosphorylation, and thus suppression of the GR pathway and steroid efficacy.

MiR-9 inhibits DEX-induced GR nuclear translocation

Because we demonstrated reduced PP2A activity concurrent with increased miR-9 expression and PP2A activity has previously been implicated in regulating GR nuclear translocation,^{39,47} we assessed the effect of ant-9 on GR localization. Previously, we demonstrated⁹ that exposure of pulmonary macrophages to DEX alone results in nuclear GR translocation, which is blocked in the presence of IFN- γ /LPS (Fig 5, A and B). Pretreatment with ant-9 restored DEX-driven GR nuclear translocation (Fig 5, A and B). Importantly, even in the

presence of DEX, ant-9 was able to neutralize IFN- γ /LPS-induced miR-9 expression and restore PP2A activity (Fig 5, C and D). Collectively, our findings demonstrate that miR-9 blocks DEX-mediated GR nuclear translocation by modulating PP2A activity, promoting steroid-resistant AHR. Blocking miR-9 reverses this effect, restoring DEX-induced GR nuclear translocation.

PP2A activity directly affects AHR

To directly determine the effect of altered PP2A activity on both steroid-resistant AHR and GR nuclear translocation, we administered AAL(S), a compound that upregulates PP2A activity. Intranasal AAL(S) administration significantly reduced airway resistance when administered with DEX (Fig 6, A) comparable with the effect of ant-9. Furthermore, AAL(S) administration to IFN- γ /LPS-stimulated pulmonary macrophages *in vitro* also restored GR nuclear translocation (Fig 6, B and C). These findings demonstrate that increased PP2A activity is sufficient to restore DEX-induced nuclear GR translocation and render AHR sensitive to steroid.

Ant-9 inhibits LPS-induced exacerbations of steroid-resistant AHR in allergen challenge models of asthma

Having identified a role for miR-9 in steroid sensitivity in our IFN- γ /LPS model, we extended our study to more complex models of steroid-resistant AHR. Mice were sensitized and

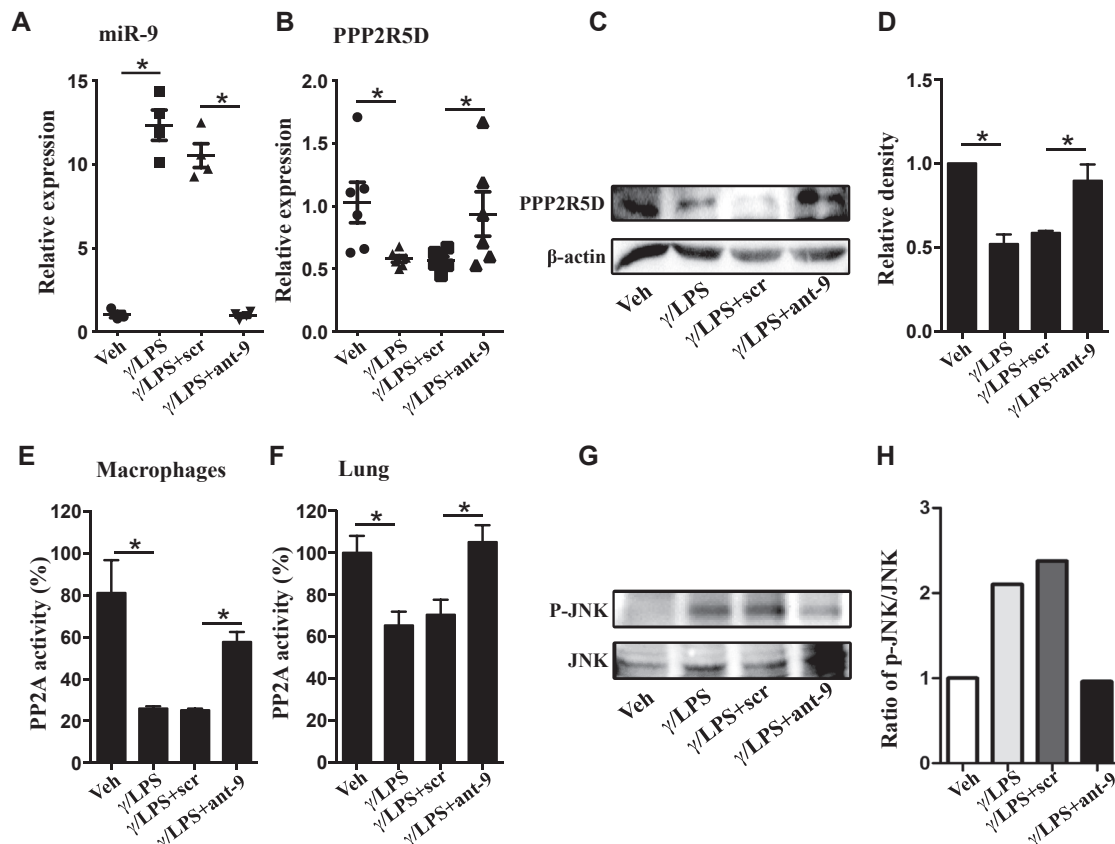


FIG 4. Targeting miR-9 restores PPP2R5D expression and PP2A activity. Pulmonary macrophages were pretreated with ant-9 or scrambled antagomir (*scr*) 12 hours before IFN- γ /LPS (γ /LPS) treatment. Samples were collected 12 hours later. **A** and **B**, MiR-9 (Fig 4, A) and PPP2R5D (Fig 4, B) transcript levels were determined by using quantitative PCR. Each dot represents the mean of an independent experiment performed in triplicate. **C** and **D**, Representative Western blot images (Fig 4, C) and densitometric assessment (Fig 4, D) for PPP2R5D protein levels. Data are representative of 3 independent experiments. **E** and **F**, PP2A catalytic activity was normalized to untreated control values in pulmonary macrophages (triplicate samples from 1 experiment representative of 3 independent experiments; Fig 4, E) and lung tissue (Fig 4, F). Data are means $\pm$ SEMs ($n = 6$ mice per group). **G** and **H**, Western blot staining of pulmonary macrophages for phosphorylated (*P-JNK*) and total JNK (Fig 4, G) and P-JNK/JNK ratios (Fig 4, H) normalized to vehicle control (*Veh*). * $P < .05$.

challenged with OVA to induce asthma-like inflammation and exposed to LPS to mimic infection and activate macrophages. LPS was administered during OVA challenge (coexposure model [allergen with infection]; Fig 7, A) or after challenge (postexposure model [infection after allergen exposure]; Fig 7, C).

In the LPS coexposure model mice were pretreated with DEX in the presence or absence of ant-9 (Fig 7, A). Measurement of lung function (1 day after the last OVA exposure) identified an LPS-induced steroid-resistant exacerbation of AHR compared with the OVA/OVA group (Fig 7, B, left panel). Administration of ant-9 plus DEX significantly reduced AHR compared with the scrambled control (Fig 7, B, right panel). Similarly, postexposure with LPS (1 week after the last OVA exposure; Fig 7, C) induced steroid-resistant AHR (Fig 7, D, left panel). In this model ant-9 plus DEX completely reduced airway responsiveness to baseline levels (Fig 7, D, right panel). In both models LPS treatments alone increased miR-9 expression, and ant-9 installation blocked this increase (see Fig E3 in this article's Online Repository at www.jacionline.org). Interestingly, LPS administration induced heightened eosinophil infiltration and mucus hypersecretion, which were also steroid resistant (see Figs E4-E6 in this

article's Online Repository at www.jacionline.org). Combined treatments with ant-9 and DEX suppressed LPS-induced heightened eosinophil infiltration but had no effect on mucus hypersecretion in the LPS coexposure model. By contrast, ant-9 and DEX together suppressed LPS-induced heightened mucus hypersecretion but had no effect on eosinophil infiltration in the LPS postexposure model. We did not observe any significant changes in collagen deposition in either model (data not shown). Collectively, these findings demonstrate that ant-9 is sufficient to restore steroid sensitivity, allowing DEX to inhibit AHR in these models.

DISCUSSION

MiRNAs are important modulators of gene expression, and their expression levels are dramatically altered during immune responses.^{9,14,31} However, the functional roles of miRNAs in the regulation of immune responses are only beginning to be identified. In the current study we assessed miRNA expression within the lung in our model of steroid-resistant AHR. We observed a synergistic upregulation of miR-9 expression by IFN- γ and LPS

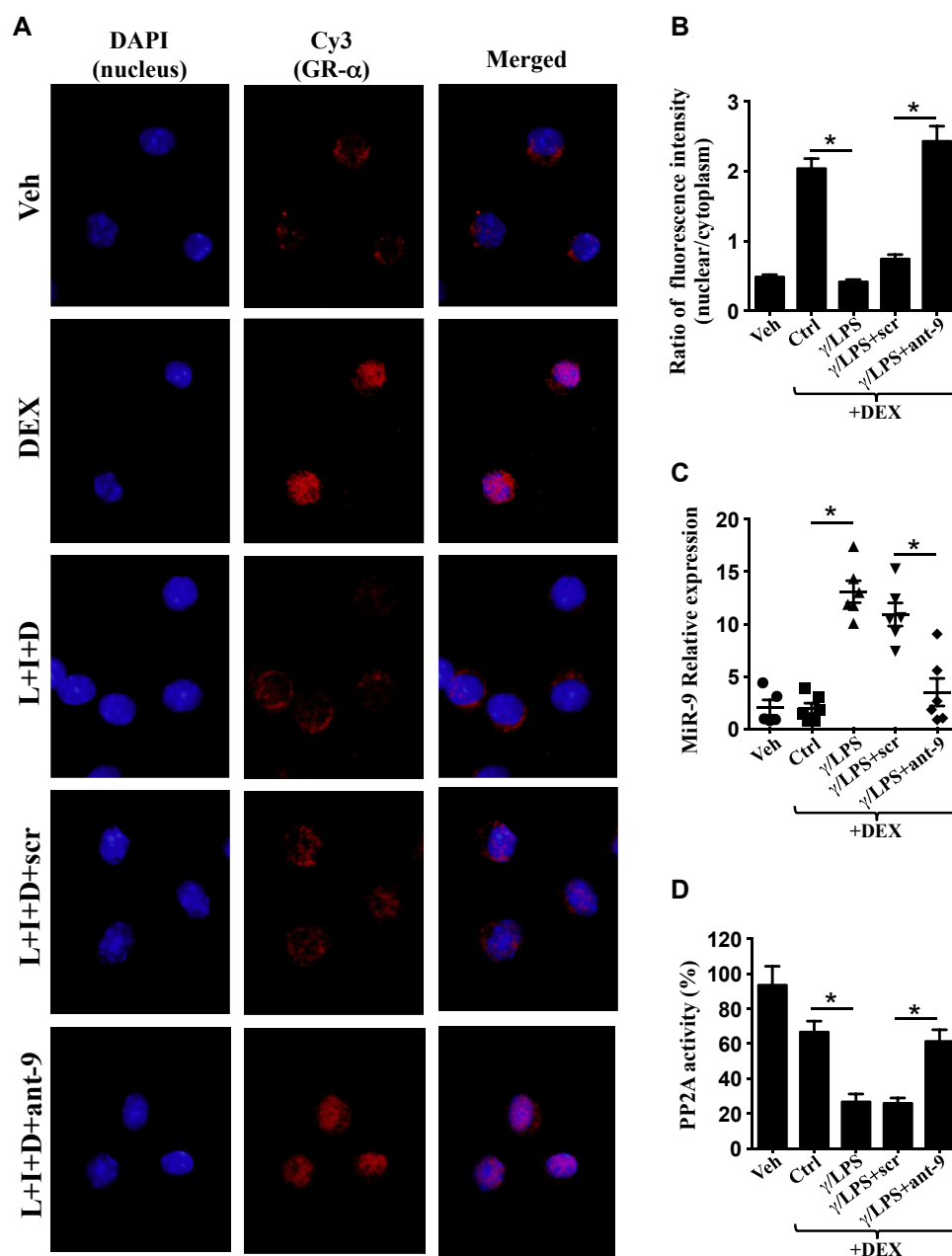


FIG 5. Inhibition of miR-9 restores DEX-induced GR nuclear translocation. Pulmonary macrophages were pretreated with ant-9 or scrambled control (*scr*) for 12 hours before IFN- γ /LPS (γ /LPS) for 11 hours and then DEX for 1 hour. **A**, GR cellular localization determined by using immunofluorescence staining. **B**, Ratio of fluorescence intensity (nuclear/cytoplasm). Assessment was performed on 8 to 10 different slide regions on 40 to 60 cells for each treatment. **C** and **D**, MiR-9 expression levels (Fig 5, C) and PP2A activities (Fig 5, D) were also determined. Values are means $\pm$ SEMs for triplicate samples from 1 experiment and representative of 3 independent experiments. D, DEX; I, IFN- γ ; L, LPS. * $P < .05$.

in lung tissue and pulmonary macrophages, linking this miRNA to the mechanism of steroid resistance. Moreover, inhibition of miR-9 restored steroid sensitivity and inhibited AHR in multiple models of steroid-resistant AHR. Taken together, our findings provide support for a model whereby activation of macrophages results in increased expression of miR-9, leading to decreased PPP2R5D expression and overall PP2A activity (Fig 8). Decreased PP2A activity results in increased JNK phosphorylation, increased GR phosphorylation, and inhibited GR nuclear translocation. Blocking miR-9 with ant-9 reverses these effects,

resulting in increased PPP2R5D expression and PP2A activity, decreased JNK phosphorylation, and restoration of DEX-induced GR nuclear translocation (Fig 8). Our findings establish a new link between miRNA and glucocorticoid sensitivity in macrophages and highlight the potential of targeting miRNA pathways for the treatment of steroid-resistant disease.

MiR-9 was initially identified as a neuronal-specific miRNA and implicated in mammalian nerve development.^{48,49} Subsequently, miR-9 was identified in nonneural diseases, with a role in regulation of insulin cell secretory function.⁵⁰ This miRNA

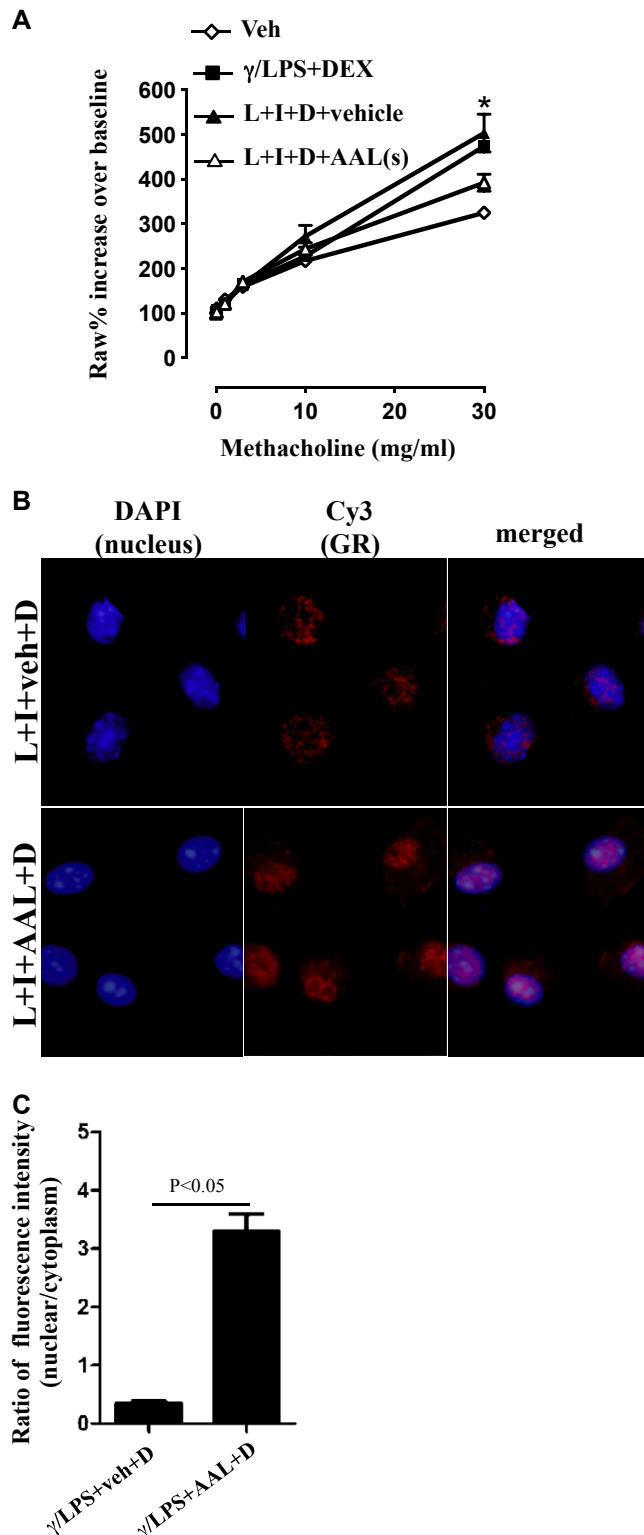


FIG 6. Increasing PP2A activity restores steroid sensitivity and GR nuclear translocation. Mice administered DEX and/or the PP2A activator AAL(S) after IFN- γ /LPS (γ /LPS) exposure are shown. **A**, Lung function was measured 12 hours later. Values are means $\pm$ SEMs ($n = 8$ to 12 mice per group). **B** and **C**, Isolated pulmonary macrophages treated with DEX with or without AAL(S) and GR cellular localization determined by means of immunofluorescence staining (Fig 6, **B**) and the ratio of fluorescence intensity (nuclear/cytoplasm; Fig 6, **C**). Assessment was performed on 8 to 10 different slide regions on 40 to 60 cells for each treatment. **D**, DEX;

has also been implicated in regulating cancer cell proliferation and metastasis and has been associated with cardiotoxicity through regulation of platelet-derived growth factor receptor β expression.⁵¹⁻⁵³ Recently, miR-9 expression was shown to be induced by high-dose (100 ng/mL) LPS in primary human polymorphonuclear neutrophils and monocytes in a myeloid differentiation primary response gene 88- and nuclear factor κ B-dependent manner.⁵⁴ Furthermore, miR-9-dependent inhibition of NF κ B1 transcription suggests that the rapid induction of miR-9 operates as a feedback control of nuclear factor κ B-dependent responses in these cells.⁵⁴ MiR-9 was also induced by LPS and *Staphylococcus aureus* enterotoxin B in bovine monocytes.⁵⁵ By contrast, in our study we demonstrate synergistic upregulation of miR-9 expression by IFN- γ /LPS in pulmonary macrophages. LPS stimulation alone (at 5 ng/mL) was not sufficient to induce miR-9 expression, but IFN- γ treatment alone enhanced expression modestly.

Glucocorticoids exert their anti-inflammatory effect through GR activation, directing downstream transcription and signal transduction. A number of possible mechanisms have been proposed to explain the development of steroid resistance, including decreased histone deacetylase activity, overexpression of proinflammatory transcription factors, and abnormal GR expression.^{6,56} Posttranscriptional modifications of the GR, such as phosphorylation, are critical for modulating GR function and might also underlie one mechanism of steroid resistance.^{57,58} GR phosphorylation impairs nuclear translocation and modifies transcriptional activity. One phosphorylation site on the human GR (Ser 226) is mainly phosphorylated by JNK.⁵⁹ JNK is activated by inflammatory stimuli, including inflammatory cytokines and LPS, and its activation results in phosphorylation of various transcription factors, promoting their transcriptional activity.⁶⁰ In our observations, IFN- γ /LPS treatment results in increased JNK phosphorylation, which is reversed after ant-9 administration. This activation of JNK might in turn lead to the observed alteration in GR nuclear translocation, mediating steroid resistance in our model.

PP2A is ubiquitously expressed and functions as a serine/threonine phosphatase.⁶¹ PP2A functions as a heterotrimeric enzyme composed of a catalytic C subunit, a structural subunit, and multiple regulatory B subunits, which regulate PP2A function by modulating substrate binding, enzyme activity, and subcellular localization.⁶² Previous studies suggest PP2A can increase GR activity through dephosphorylation of JNK and also regulate GR translocation to the nucleus.^{39,47} Furthermore, in an animal model of virus-induced exacerbation of asthma, PP2A activity was significantly decreased, whereas synthetic upregulation of PP2A activity resulted in reduced AHR.⁶³

PPP2R5D is a critical PP2A regulatory subunit, and its expression and phosphorylation regulate the catalytic activity of PP2A.⁴³⁻⁴⁶ To clarify the mechanism behind miR-9 function, we performed a dual luciferase reporter assay and demonstrated miR-9-mediated inhibition of PPP2R5D and PPP2R2A expression. Further investigations in pulmonary macrophages demonstrated that ant-9-mediated inhibition of miR-9 restored expression of PPP2R5D but not PPP2R2A. A PP2A activity assay confirmed that IFN- γ /LPS stimulation resulted in decreased catalytic

← I, IFN- γ ; L, LPS; Veh, vehicle control. * $P < .05$, γ /LPS+DEX, L+I+D+vehicle versus vehicle control, L+I+D+AAL(s).

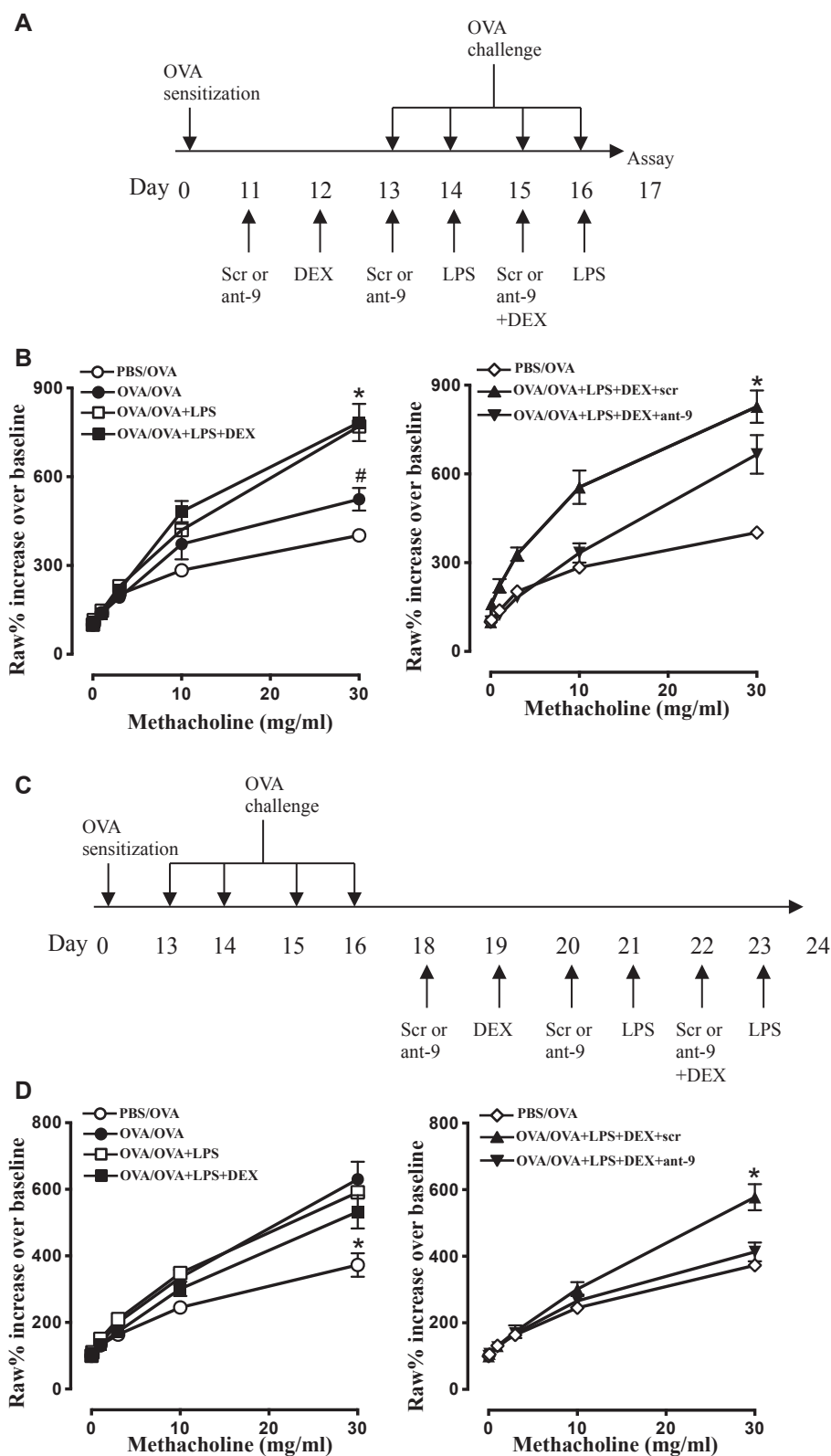


FIG 7. Inhibition of miR-9 restores steroid sensitivity, blocking the development of AHR in allergen-induced models of steroid-resistant AHR. **A** and **C**, Schematic representation of coexposure (Fig 7, A) and postexposure (Fig 7, C) models of LPS administration indicating timing of OVA sensitization, challenges, and DEX/ant-9 treatments. **B** and **D**, Lung function was assessed on day 17 after sensitization (Fig 7, B) and day 24 after sensitization (Fig 7, D), respectively. Values are means $\pm$ SEMs ($n = 10$ mice per group). Raw, Airway resistance. * $P < .05$ versus other groups. # $P < .05$, OVA/OVA versus OVA/LPS and OVA/LPS+DEX.

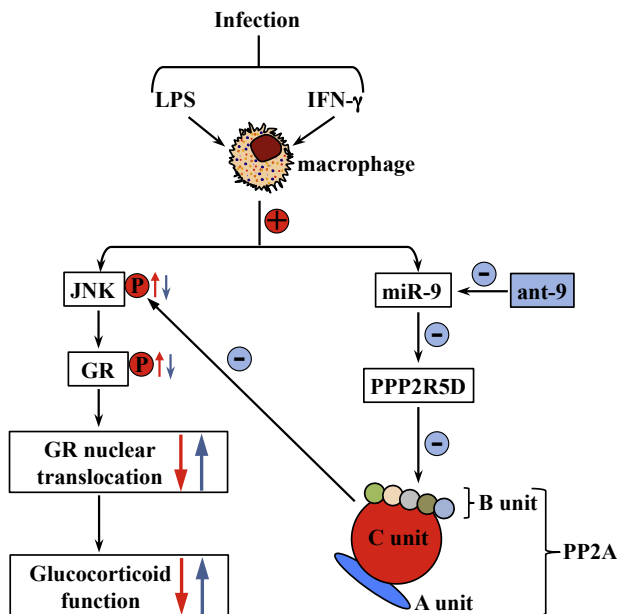


FIG 8. Schematic representation of miR-9's effects on glucocorticoid function in pulmonary macrophages after IFN- γ /LPS exposure. miR-9 reduces PP2A activity by reducing PPP2R5D levels, thus downregulating GR nuclear translocation and inhibiting glucocorticoid function. Ant-9 recovers PP2A activity, leading to improved glucocorticoid function. $\oplus$, Increased; $\ominus$, decreased; $\textcircled{P}$, phosphorylation. Red arrow, miR-9 effect; blue arrow, ant-9 effect.

PP2A activity both *in vitro* and *in vivo*, whereas inhibition of miR-9 restored activity. These results suggested that miR-9 exerts its effect by regulating the activity of PP2A through modulation of the regulatory PPP2R5D subunit.

PP2A activity is significantly reduced in multiple inflammatory diseases, and reactivation with various activators, such as AAL(S) or FTY-720, has been shown to abrogate the development of inflammation and AHR in mouse models of asthma.⁶³⁻⁶⁵ AAL(S) is a FTY720 analogue that reactivates PP2A activity through inhibition of SET oncoprotein (I2PP2A).^{63,66-68} It is believed that SET inhibits PP2A activity through direct binding with the catalytic subunit C of PP2A.⁶⁹ FTY720 prevents SET-PP2A binding through its direct association with SET in a hydrophobic pocket in its C-terminal region.⁶⁷ Therefore it appears that miR-9 and AAL(s) regulate PP2A activity, although through different mechanisms.

Thus our results indicate that altered PP2A activity plays a central role in our model of steroid-resistant AHR. Indeed, previous studies have also demonstrated a critical role for PP2A in the regulation of GR phosphorylation and nuclear translocation.^{39,47} In PBMCs from patients with severe asthma, PP2A expression and activity were significantly reduced compared with values in healthy volunteers, suggesting a potential mechanism for PP2A activity in regulating development of steroid resistance in these patients.³⁹ In our study ant-9 treatment restored PP2A activity but also concomitantly reduced JNK phosphorylation, resulting in restored DEX-induced GR nuclear translocation.

Our findings also reveal that localized targeting of miR-9 within the lung with ant-9 was sufficient to restore DEX

sensitivity and reduce AHR in a number of models of steroid-resistant AHR. Interestingly, the observed reduction in AHR after ant-9 administration occurred only in the presence of DEX treatment, with no observed changes in immune cell infiltration to the lung. These results suggest that upregulation of miR-9 blocks the normal glucocorticoid-induced signaling pathway and that targeting miR-9 could serve as a novel approach to restore glucocorticoid responsiveness without altering the immune response.

In summary, our data suggest that increased miR-9 expression contributes to steroid-resistant AHR by decreasing PPP2R5D levels and the subsequent PP2A activity. Blocking miR-9 function restores steroid sensitivity and might serve as a novel approach for the treatment of steroid-resistant AHR.

Key messages

- IFN- γ and LPS synergistically induce miR-9 expression in lung tissue and primary macrophages, and miR-9 expression is increased in sputum cells of patients with neutrophilic asthma.
- Increased miR-9 expression reduces PPP2R5D levels, attenuates PP2A activity, and blocks DEX-induced GR nuclear translocation.
- Blocking miR-9 function restores PP2A activity and steroid-sensitive AHR.

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