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*

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.

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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 μL 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 μg per mouse; PeproTech, Rocky Hills, NJ), LPS (50 ng per mouse, Sigma-Aldrich), or IFN-γ plus LPS (IFN-γ/LPS; 1.5 μg plus 50 ng per mouse) in 50 μL 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 μg 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 μL 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 described[14] 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 hydroxyprolinol-linked cholesterol. Antagomir-9 (ant-9; miR-9 sequence-specific antagomir) or scrambled antagomir control (50 μg per mouse) were administered intratracheally 3 times, as indicated in the respective figure. Quantitative assessment of