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Andrographolide Restores Steroid Sensitivity To Block Lipopolysaccharide/IFN- γ -Induced IL-27 and Airway Hyperresponsiveness in Mice

Wupeng Liao,* W. S. Daniel Tan,* and W. S. Fred Wong*,[†]

LPS and IFN- γ alone or in combination have been implicated in the development of steroid resistance. Combined LPS/IFN- γ strongly upregulates IL-27 production, which has been linked to steroid-resistant airway hyperresponsiveness (AHR). Andrographolide, a bioactive molecule isolated from the plant *Andrographis paniculata*, has demonstrated anti-inflammatory and antioxidant properties. The present study investigated whether andrographolide could restore steroid sensitivity to block LPS/IFN- γ -induced IL-27 production and AHR via its antioxidative property. The mouse macrophage cell line Raw 264.7, mouse primary lung monocytes/macrophages, and BALB/c mice were treated with LPS/IFN- γ , in the presence and absence of dexamethasone and/or andrographolide. Levels of IL-27 in vitro and in vivo were examined and mouse AHR was assessed. Dexamethasone alone failed to inhibit LPS/IFN- γ -induced IL-27 production and AHR in mice. Andrographolide significantly restored the suppressive effect of dexamethasone on LPS/IFN- γ -induced IL-27 mRNA and protein levels in the macrophage cell line and primary lung monocytes/macrophages, mouse bronchoalveolar lavage fluid and lung tissues, and AHR in mice. LPS/IFN- γ markedly reduced the nuclear level of histone deacetylase (HDAC)2, an essential epigenetic enzyme that mediates steroid anti-inflammatory action. LPS/IFN- γ also decreased total HDAC activity but increased the total histone acetyltransferase/HDAC activity ratio in mouse lungs. Andrographolide significantly restored nuclear HDAC2 protein levels and total HDAC activity, and it diminished the total histone acetyltransferase/HDAC activity ratio in mouse lungs exposed to LPS/IFN- γ , possibly via suppression of PI3K/Akt/HDAC2 phosphorylation, and upregulation of the antioxidant transcription factor NF erythroid-2-related factor 2 level and DNA binding activity. Our data suggest that andrographolide may have therapeutic value in resensitizing steroid action in respiratory disorders such as asthma. *The Journal of Immunology*, 2016, 196: 4706–4712.

Recent studies have highlighted the role of innate immune cells including pulmonary macrophages in more severe types of asthma, which are often associated with steroid-resistant airway hyperresponsiveness (AHR) (1, 2). Acute exacerbations of asthma, often triggered by respiratory bacterial or viral infections, are critical clinical problems for asthma management and treatment. High levels of LPS and Th1 cytokine IFN- γ have been detected in the airways of asthmatics, which are often correlated with asthma severity (3–7). LPS and IFN- γ are two key mediators for exacerbative inflammatory responses and the decrease in sensitivity to steroid in experimental models of asthma (7–9). Macrophage-derived IL-27 has been shown to be upregulated by LPS/IFN- γ and mediate LPS/IFN- γ -induced steroid-resistant AHR, as neutralizing IL-27 Ab significantly attenuated this response (10).

Corticosteroid is the first-line anti-inflammatory drug treatment for asthma. Corticosteroid-bound glucocorticoid receptor (GR) translocates into the nucleus, not only to induce anti-inflammatory gene expression in a process called transactivation, but also to suppress proinflammatory gene transcription by antagonizing transcription factor NF- κ B via transrepression (11). To achieve optimum transrepression by GR, it recruits a transcription corepressor histone deacetylase (HDAC)2 to the NF- κ B transcriptome complex, reversing the unwrapping process of DNA for NF- κ B-regulated gene transcription (12). Oxidative stress results in decreased level and impaired activity of HDAC2, which has been proposed to play a critical role in steroid insensitivity in asthmatic and chronic obstructive pulmonary disease (COPD) patients (12). Thus, agents that possess an antioxidative property have the potential to restore HDAC2 levels, which may then resensitize steroid efficacy and anti-inflammatory activities.

Andrographolide is a labdane diterpene lactone bioactive molecule isolated from the *Andrographis paniculata* plant (13). *A. paniculata* has long been used as herbal medicine for the prevention and treatment of upper respiratory tract infection in Asian countries and in Scandinavia (14). Andrographolide has been shown to possess various biological activities, including antiviral (15), anticancer (13, 16), anti-inflammatory (17, 18) and antioxidative (15, 19, 20) activities.

In this study, we hypothesized that andrographolide could restore steroid sensitivity via its antioxidative property. Our findings reveal that combined LPS/IFN- γ -induced IL-27 production by macrophages was insensitive to the transrepressive action of steroids. We observed, to our knowledge for the first time, that andrographolide could restore steroid sensitivity to block LPS/IFN- γ -upregulated IL-27 levels in mouse lung monocytes/macrophages, as well as LPS/IFN- γ -induced AHR in mice, probably through inhibition of

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; GR, glucocorticoid receptor; HAT, histone acetyltransferase; HDAC, histone deacetylase; Nrf2, NF erythroid-2-related factor 2; qPCR, quantitative PCR; TBP, TATA box binding protein.

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the PI3K/Akt/HDAC2 pathway, activation of antioxidative transcription factor NF erythroid-2-related factor 2 (Nrf2), and restoration of HDAC2 level and total HDAC activity. Our findings support a novel therapeutic value for andrographolide in resensitizing steroid for the treatment of severe asthma and asthma exacerbation.

Materials and Methods

Materials

Mouse monocyte/macrophage cell line Raw 264.7 was from American Type Culture Collection (Manassas, VA). Dexamethasone 21-phosphate disodium salt ($\geq 98\%$), andrographolide ($\geq 98\%$), LPS (*Escherichia coli* 0111:B4), acetyl- β -methylcholine chloride (methacholine), and DMSO ($\geq 99\%$ cell culture grade) were obtained from Sigma-Aldrich (St. Louis, MO). DMEM, FBS, M-PER mammalian protein extraction reagent, and Pierce protease and phosphatase inhibitor mini tablets came from Thermo Scientific (Rockford, IL). Mouse IFN- γ (carrier-free) recombinant protein, anti- β -actin, anti-HDAC2, anti-Nrf2, anti-Akt, anti-phospho-Akt (Ser⁴⁷³), anti-p70S6K, and anti-phospho-p70S6K (Ser³⁸⁹) Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-TATA box binding protein (TBP) and anti-phospho-HDAC2 (Ser³⁹⁴) Abs were from Abcam (Cambridge, U.K.). RNAlater solution, SYBR Green qPCR MasterMix, collagenase type I, and DNase I were purchased from Life Technologies (Carlsbad, CA). RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized using qScript cDNA SuperMix (Quanta, Gaithersburg, MD). Percoll was obtained from GE Healthcare (Buckinghamshire, U.K.). Mouse IL-27p28/IL-30 DuoSet ELISA kit was generated by R&D Systems (Minneapolis, MN). Nuclear extraction kit was purchased from Active Motif (Carlsbad, CA). Nrf2 transcription factor assay kit was from Cayman Chemical (Ann Arbor, MI). Histone acetyltransferase (HAT) activity assay kit and Fluor de Lys-Green HDAC2 fluorometric drug discovery assay kit came from Enzo Life Sciences (Farmingdale, NY). All primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA).

Animals

Female BALB/c mice 6- to 8-wk-old (InVivos, Singapore) were maintained in a 12-h light/dark cycle with food and water available ad libitum. Animal experiments were performed according to institutional guidelines of the Animal Care and Use Committee of the National University of Singapore.

Administration of LPS, IFN- γ , dexamethasone, or andrographolide into mice

Dexamethasone (1 mg/kg in PBS) (8, 10), andrographolide (1 mg/kg in 2% DMSO), or vehicle (2% DMSO) in 0.1 ml PBS was administered by daily i.p. injection for 3 consecutive days. One hour after the last injection, 50 ng LPS and/or 1.5 μ g mouse rIFN- γ in 50 μ l PBS was instilled intratracheally (8, 10). Sixteen hours later, mice were anesthetized for AHR measurement or sacrificed to collect lung samples for various biochemical analyses.

Bronchoalveolar lavage fluid and lung tissue harvest

Tracheotomy was performed and a cannula was inserted into the mouse trachea. Ice-cold PBS (0.5 ml $3\times$) was instilled into the lungs, and bronchoalveolar lavage (BAL) fluid was collected and kept at -80°C for analysis. Mouse lungs were excised. The left lobe was stored in RNAlater solution and right lobes were snap frozen in liquid nitrogen and then stored at -80°C for protein isolation.

Measurements of AHR

Mice were anesthetized, and tracheotomy and cannulation were performed (21). Briefly, the trachea was intubated with a cannula that was connected to the pneumotach, ventilator, and nebulizer. Lung resistance in response to PBS control and nebulized methacholine (2.5, 5, and 10 mg/ml) were recorded using a whole-body plethysmography chamber and the FinePointe data acquisition and analysis software (Buxco, Wilmington, NC), as described (21). Results are expressed as a percentage of the respective basal values in response to PBS.

Lung alveolar and parenchymal macrophage/monocyte preparations

Lung alveolar macrophages were obtained by pooling and pelleting the BAL fluids from 20 naive mice. The cell morphology was assessed by

Giemsa staining, and $>97\%$ of BAL cells from naive mice were identified as macrophage morphology. Mouse whole lung was perfused with 5 ml PBS and minced into 2- to 3-mm pieces. Full DMEM medium containing type I collagenase (50 μ g/ml) and DNase I (100 U/ml) was used to isolate lung total cell suspension. Lung parenchymal mononuclear cells were further obtained by using Percoll density gradient centrifugation. Briefly, single cells from digested lungs were resuspended in Percoll gradient (40 over 80%) and centrifuged at $800 \times g$ for 30 min. Mononuclear cells were collected from the interphase and washed with PBS for further culture and analysis. Mononuclear cell suspensions were removed from the culture media, and the adherent cell morphology was identified mainly as monocytes/macrophages by Giemsa staining.

Cell culture and treatment in vitro

The mouse monocyte/macrophage cell line Raw 264.7 or isolated primary mouse lung alveolar and parenchymal monocytes/macrophages were cultured in DMEM medium supplemented with 10% FBS. All cell cultures were maintained in a humidified 37°C , 5% CO_2 incubator. The cells were starved with 1% FBS overnight before treatment with various concentrations of andrographolide (0.05% DMSO as vehicle) and/or dexamethasone, as well as 50 ng/ml LPS and 150 ng/ml mouse IFN- γ for indicated time points.

IL-27 ELISA

A mouse IL-27p28/IL-30 DuoSet ELISA kit was used to measure the IL-27 level in cell culture media and mouse BAL fluid according to the manufacturer's instructions. Briefly, 96-well BD Falcon ELISA plates were coated overnight at 4°C with anti-mouse IL-27p28 capture Ab. After washing and blocking, BAL fluid, culture media, or standards were added and incubated for 2 h. The biotinylated anti-mouse IL-27p28 detection Ab, mixed with enzyme reagent streptavidin-HRP conjugate, was added and incubated for 1 h at room temperature. The plates were washed and developed with a tetramethylbenzidine peroxidase EIA substrate kit (BD Biosciences). The reaction was stopped with stopping solution (1 M H_2SO_4) and the OD was read at 450 nm with a reference of 600 nm on an Infinite 200 microplate reader (Tecan, Männedorf, Switzerland).

RNA extraction, reverse transcription, and quantitative PCR

Total RNA was extracted from cells or homogenized mouse lung tissues using an RNeasy mini kit according to the manufacturer's instructions. cDNAs were synthesized using qScript cDNA SuperMix. cDNA synthesis was performed by a gradient thermal cycler (Biometa, Goettingen, Germany). Quantitative PCR (qPCR) was performed with SYBR Green qPCR MasterMix as a detection dye in an ABI 7500 real-time PCR machine presented as fold differences over the controls by the $2^{-\Delta\Delta\text{Ct}}$ method. Mouse β -actin gene *Actb* was used as an endogenous control. The primer pairs are listed as follows: mouse *Actb*, forward, 5'-TCA TGA AGT GTG ACG TTG ACA TCC G-3', reverse, 5'-CCT AGA AGC ATT TGC GGT GCA CGA TG-3'; mouse *Il27p28*, forward, 5'-CTG TTG CTG CTA CCC TTG CTT-3', reverse, 5'-CAC TCC TGG CAA TCG AGA TTC-3'.

Total lysate, nuclear fractionation, and immunoblotting

Raw cells and frozen lung tissues were lysed in M-PER mammalian protein extraction reagent containing Pierce protease and phosphatase inhibitor mini tablets for total protein extraction. Cytoplasmic and nuclear proteins were extracted from cells using a nuclear extraction kit. Protein extracts were separated by 10% SDS-PAGE, and immunoblots were probed with anti-TBP, anti-Nrf2, anti- β -actin, anti-HDAC2, anti-phospho-HDAC2 (Ser³⁹⁴), anti-Akt, anti-phospho-Akt (Ser⁴⁷³), anti-p70S6K, and anti-phospho-p70S6K (Ser³⁸⁹) Abs. Band intensity was quantitated using ImageJ software (National Institutes of Health).

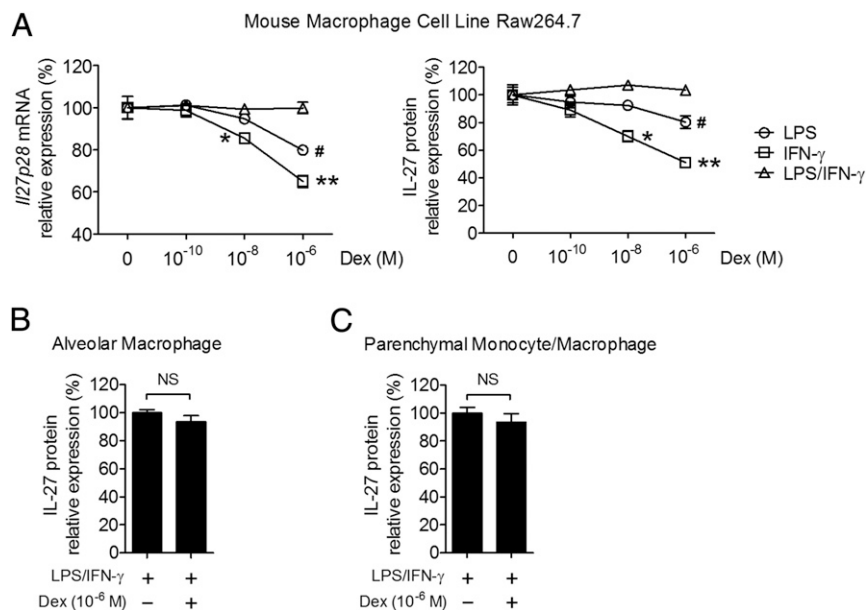
Total HAT and HDAC activity assays

Nuclear lysates were obtained from mouse lung tissues. Total HAT activity and HDAC activity were assayed using 2 and 1 μ g nuclear lysates, respectively, according to the manufacturer's instructions. The colorimetric or fluorescent readings were normalized to the PBS controls. Total HAT/HDAC activity ratio was calculated using the normalized values.

Nrf2 transcription factor binding activity assay

Mouse lung tissue nuclear lysates (20 μ g) were assayed for Nrf2 DNA binding activity according to the manufacturer's instructions. DNA bind-

FIGURE 1. Induction of steroid-resistant IL-27 production by LPS/IFN- γ in mouse monocytes/macrophages. **(A)** Relative IL-27 mRNA and protein expression levels upon 10^{-10} , 10^{-8} , and 10^{-6} M dexamethasone pretreatment, in response to LPS, IFN- γ , and combined LPS/IFN- γ in the mouse macrophage cell line Raw 264.7. Relative IL-27 protein expression levels upon 10^{-6} M dexamethasone pretreatment, in response to combined LPS/IFN- γ in mouse primary alveolar **(B)** and parenchymal **(C)** monocytes/macrophages, are shown. Data are representative of three to four independent experiments. Values are expressed as mean $\pm$ SEM. For each curve presented in (A), the relative IL-27 expression percentage is normalized over its respective stimulus control. * $p < 0.05$, ** $p < 0.01$ compared with individual stimulus control.



ing activity of nuclear Nrf2 protein was determined by absorbance at 450 nm on an Infinite 200 microplate reader.

Statistical analysis

The data shown are presented as means $\pm$ SEM of n animals or n cultured samples. One-way ANOVA followed by a Dunnett post hoc test was used for multiple treatment group comparisons with GraphPad Prism 5.0 software. Significance levels were set at $p < 0.05$.

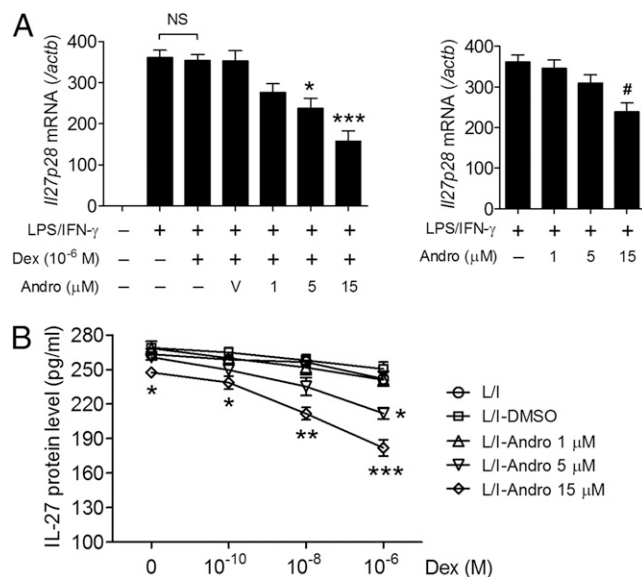


FIGURE 2. Restoration of the suppressive effect of dexamethasone against LPS/IFN- γ -induced IL-27 by andrographolide in Raw 264.7 cells. **(A)** LPS/IFN- γ -stimulated IL-27 mRNA fold change upon increasing doses of andrographolide (1, 5, and 15 μ M) or vehicle control, in the presence and absence of 10^{-6} M dexamethasone. For qPCR, mouse β -actin gene *Actb* was used as an endogenous control. **(B)** LPS/IFN- γ -stimulated IL-27 protein levels upon increasing doses (1, 5, and 15 μ M) of andrographolide or vehicle control, in the presence and absence of increasing doses of dexamethasone (10^{-10} , 10^{-8} , and 10^{-6} M). Data are representative of three to four independent experiments. Values are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with vehicle control. # $p < 0.05$ compared with stimulus control.

Results

LPS/IFN- γ -induced IL-27 production is steroid resistant

Host defense pathways induced by LPS, IFN- γ , and LPS/IFN- γ -stimulated IL-27 have been implicated in causing steroid-refractory AHR (7–10). We first examined the anti-inflammatory effects of the corticosteroid dexamethasone on the expression of IL-27 mRNA and protein upon exposure to LPS, IFN- γ , or combined LPS/IFN- γ in the mouse macrophage cell line Raw 264.7. Combined LPS/IFN- γ treatment resulted in insensitivity to the suppressive action of dexamethasone (10^{-10} , 10^{-8} , 10^{-6} M) on the production of IL-27 mRNA and protein (Fig. 1A), as compared with either LPS or IFN- γ exposure alone. Next, primary alveolar macrophages and parenchymal monocytes/macrophages, isolated from naive BALB/c mice, were stimulated with combined LPS/IFN- γ in the presence or absence of 10^{-6} M dexamethasone. IL-27 production was found totally unresponsive to the inhibitory effect of dexamethasone (Fig. 1B, 1C).

Andrographolide restores the suppressive effect of dexamethasone against LPS/IFN- γ -induced IL-27 production

LPS/IFN- γ combination induced a >350 -fold change of IL-27 subunit p28 in the Raw 264.7 cell line, which was almost completely resistant to the suppressive effect of 10^{-6} M dexamethasone. Andrographolide exhibited a concentration-dependent restoration of dexamethasone-mediated suppression of IL-27p28 mRNA, with significant suppression observed at 5 and 15 μ M, whereas andrographolide alone only showed minimal inhibitory effect on IL-27p28 mRNA even at the high dose of 15 μ M (Fig. 2A). Additionally, andrographolide exhibited concentration-dependent facilitation of IL-27 protein inhibition in response to dexamethasone (Fig. 2B). All treatments did not show any significant cytotoxic effects as measured by MTS assay (data not shown).

Similar to what has been observed in the Raw 264.7 cell line, 10^{-6} M dexamethasone alone failed to inhibit LPS/IFN- γ -induced IL-27 protein production in isolated mouse alveolar macrophages or parenchymal monocytes/macrophages. However, addition of andrographolide simultaneously to dexamethasone suppressed IL-27 protein level in a synergistic manner, as compared with the effects of andrographolide alone (Fig. 3). In contrast to alveolar macrophages (Fig. 3A), parenchymal monocytes/macrophages (Fig. 3B) were found to be more sensitive to the restoration effect

of 1 μ M andrographolide on dexamethasone-mediated inhibition of LPS/IFN- γ -induced IL-27 production. All treatments did not show any significant cytotoxic effects as measured by MTS assay (data not shown).

Andrographolide suppresses the PI3K/Akt/HDAC2 pathway and activates Nrf2

A number of possible mechanisms have been proposed to explain the development of steroid resistance, including abnormal GR expression or translocation, overexpression of proinflammatory transcription factors, and decreased transcription corepressor HDAC2 level or activity, among which HDAC2 is indispensable to achieve the maximal corticosteroid transrepression activity (22). In Raw 264.7 cells, we found that nuclear HDAC2 protein level decreased by >40% at 16 h after LPS/IFN- γ treatment (Fig. 4A). Andrographolide concentration-dependently restored the nuclear HDAC2 protein level at the concentration as low as 5 μ M (Fig. 4B). Additionally, andrographolide also increased the level of nuclear Nrf2, a critical redox-sensitive transcription factor, in a concentration-dependent manner (Fig. 4B). It has been reported that PI3K/Akt, NF- κ B, and p38 MAPK pathways could be involved in steroid resistance (23–25). LPS/IFN- γ drastically stimulated phosphorylation of PI3K downstream targets Akt and p70S6K (Fig. 4C). Andrographolide significantly inhibited p-Akt and p-p70S6K, at the concentration as low as 5 μ M (Fig. 4C). Downregulation of HDAC2 level involves posttranslational modifications such as serine phosphorylation and proteasomal degradation, and the phosphorylation of HDAC2 is dependent on PI3K/Akt signaling pathway (26–28). We also observed a corresponding suppression of serine

phosphorylation of LPS/IFN- γ -induced HDAC2 by andrographolide in a concentration-dependent manner (Fig. 4D).

Andrographolide restores steroid sensitivity to block LPS/IFN- γ -induced IL-27 and AHR in vivo

It has been reported that intratracheal administration of combined LPS/IFN- γ induced a robust AHR, as compared with either LPS or IFN- γ given alone (8). As shown in Fig. 5A, combined LPS/IFN- γ intratracheal instillation for 16 h resulted in a dramatic increase in AHR to increasing doses of methacholine. However, LPS or IFN- γ exposure alone did not promote any significant change in AHR. Pretreatment with 1 mg/kg dexamethasone given i.p. for 3 d had minimal effects on LPS/IFN- γ -induced AHR. Interestingly, low-dose andrographolide (1 mg/kg) by itself had no effect on AHR, but it facilitated dexamethasone in blocking LPS/IFN- γ -induced AHR (Fig. 5B). Furthermore, andrographolide restored steroid sensitivity to block LPS/IFN- γ -induced IL-27p28 mRNA in mouse lung tissues (Fig. 5C) and IL-27 protein levels from mouse BAL fluids (Fig. 5D).

Andrographolide restores HDAC2 protein level and total HDAC activity and enhances Nrf2 DNA binding activity in LPS/IFN- γ -exposed mouse lung

Intratracheal LPS/IFN- γ administration markedly decreased HDAC2 protein level (Fig. 6A) and total HDAC activity (Fig. 6B) in mouse lung tissues. Andrographolide significantly restored HDAC2 protein level and total HDAC activity in LPS/IFN- γ -exposed lungs. Acetylation of histones by transcriptional coactivator HAT leads to chromatin opening for inflammatory gene transcription. It has been reported that the total HAT/HDAC activity ratio is elevated in lungs from severe asthmatics (29). In LPS/IFN- γ -treated mouse lungs, we observed a significant increase in total HAT/HDAC activity ratio. Andrographolide significantly reduced the total HAT/HDAC activity ratio, contributing its steroid resensitization capability (Fig. 6C). As a potent Nrf2 inducer, andrographolide also markedly augmented nuclear Nrf2 transcription factor DNA binding activity in mouse lung tissues (Fig. 6D).

Discussion

LPS and IFN- γ alone or in combination have been implicated in the development of steroid resistance. More specifically, combined LPS/IFN- γ -induced IL-27 production has been linked to steroid-resistant AHR (7–10). A recent report has characterized and differentiated severe asthma from mild-moderate asthma by high level of IFN- γ in lung tissues and BAL fluid, where IFN- γ was found to promote steroid-refractory AHR (7). In this study, combined LPS/IFN- γ induced steroid resistance in IL-27 production and methacholine-induced AHR, and andrographolide was able to restore sensitivity of dexamethasone in inhibiting IL-27 production and AHR, probably via upregulation of nuclear HDAC2 level and total HDAC activity.

IL-27 is a heterodimeric cytokine that contains two subunits, an EBV-induced gene 3 and a p28 chain. It is produced by activated monocytes/macrophages and dendritic cells. The induction of both IL-27p28 and EBV-induced gene 3 is dependent on cellular signaling through TLR4/MyD88 and transcription factor IRF-1 and NF- κ B (30, 31). BAL fluid and serum IL-27 levels were elevated in acute lung injury/acute respiratory distress syndrome patients (32). Markedly higher sputum or plasma concentrations of IL-27 were found in patients with COPD and COPD exacerbation (33, 34). In asthma, IL-27 was initially reported as an immunomodulatory molecule to suppress Th2 response in a way that it antagonizes Th2 signaling by potentiating Th1 response (35, 36). However, IL-27 levels also surged in induced sputum (10) and BAL fluid (37)

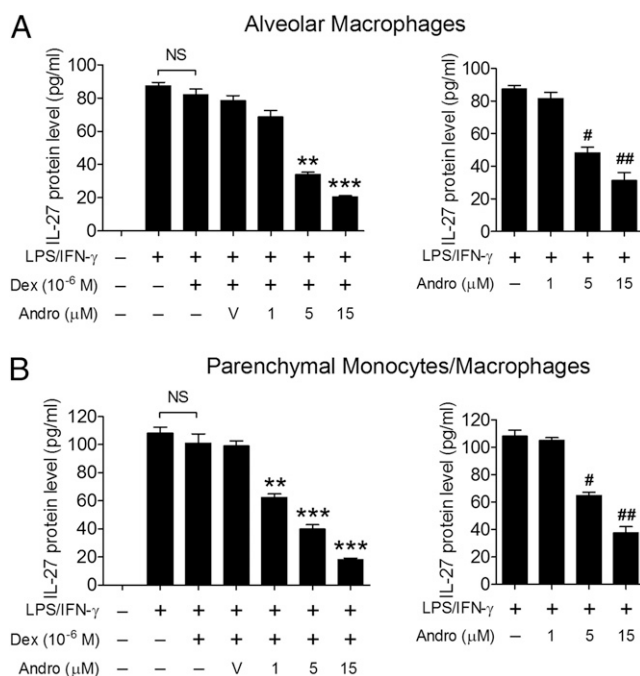


FIGURE 3. Restoration of the suppressive effect of dexamethasone against LPS/IFN- γ -induced IL-27 production by andrographolide in mouse lung monocytes/macrophages. LPS/IFN- γ -stimulated IL-27 protein levels upon increasing doses (1, 5, and 15 μ M) of andrographolide or vehicle control, in the presence and absence of 10^{-6} M dexamethasone in mouse lung alveolar (A) and parenchymal (B) monocytes/macrophages, are shown. Data are representative of three independent experiments. Values are expressed as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with vehicle control. # p < 0.05, ## p < 0.01 compared with stimulus control.

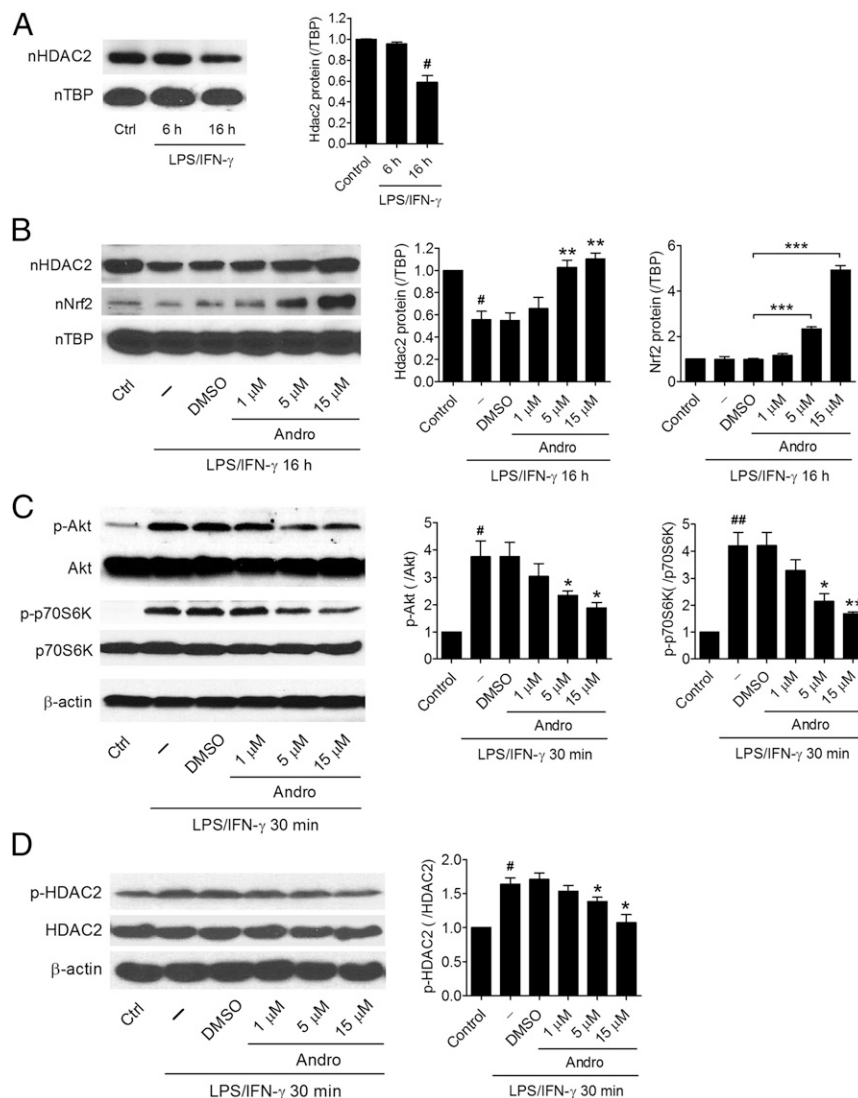


FIGURE 4. Suppression of the PI3K/Akt/HDAC2 pathway and activation of Nrf2 in Raw 264.7 cells by andrographolide. **(A)** Immunoblot and corresponding quantification of nuclear HDAC2 protein level upon LPS/IFN- γ stimulation for indicated time points. **(B)** Immunoblots and corresponding quantifications of nuclear HDAC2 and Nrf2 levels in response to increasing doses (1, 5, and 15 μ M) of andrographolide or vehicle control after stimulation with LPS/IFN- γ for 16 h. **(C)** Akt, p70S6K, and **(D)** HDAC2 phosphorylation in response to increasing doses (1, 5, and 15 μ M) of andrographolide or vehicle control after stimulation with LPS/IFN- γ for 30 min. TBP and β -actin were used as nuclear and total protein loading controls, respectively. Band intensity was quantified using ImageJ software. Data are representative of three to four independent experiments. Values are expressed as mean $\pm$ SEM. * p < 0.05, ** p < 0.01 compared with vehicle control. # p < 0.05, ## p < 0.01 compared with unstimulated control.

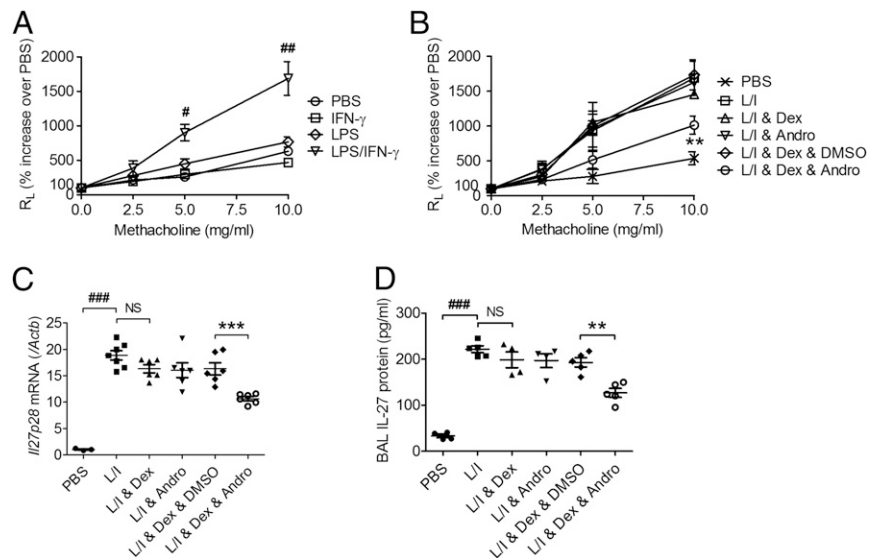
from steroid-refractory asthmatics, and in combination with the Th2/CCL26 signature, IL-27 could identify a more severe asthma phenotype (38). Although the exact function of increased IL-27 level in patients remains to be determined, an essential role of IL-27 in a mouse model of LPS/IFN- γ -induced steroid-resistant AHR has been demonstrated, as depletion of IL-27 by neutralizing Ab abolished induced AHR (10). Therefore, any strategy to restore steroid sensitivity in inhibiting IL-27 level is highly desirable.

Andrographolide is best known for its anti-inflammatory activity as an NF- κ B inhibitor. It binds specifically to the DNA binding subunit p50 at Cys⁶² residue of the NF- κ B complex, preventing the complex from binding to DNA for gene transcription (39). It has been shown that andrographolide suppressed LPS-induced p65 phosphorylation, p65 nuclear translocation, and NF- κ B DNA binding activity in mouse MEL-12 epithelial cells and Raw 264.7 cells (40, 41). However, in the present study andrographolide could not inhibit combined LPS/IFN- γ -stimulated p65 phosphorylation in the Raw 264.7 cell line (data not shown). This might be due to additional cellular signals being triggered off by IFN- γ (31). Instead, we found that andrographolide dephosphorylated LPS/IFN- γ -stimulated p-Akt and its downstream p-p70S6 kinase, at the dose as low as 5 μ M. This is not unexpected, as andrographolide has been documented to suppress the activated

PI3K/Akt pathway under inflammatory conditions (42, 43). Paradoxically, in resting cells, andrographolide functions as an inducer of the PI3K/Akt pathway to facilitate nuclear translocation of Nrf2 (20). This completely discrete action of andrographolide displayed between resting and inflammatory conditions warrants further investigation.

Intact HDAC2 function is indispensable in mediating the trans-repression action of steroids to modulate transcriptional activity of NF- κ B and AP-1 (22). Elevated HAT activity and impaired HDAC2 protein expression and activity have been reported in lung tissues, alveolar macrophages, and PBMCs from COPD and severe asthma patients (29, 44, 45). In the present study, we observed a major drop in nuclear HDAC2 level and total HDAC activity and an increase of the total HAT/HDAC activity ratio upon LPS/IFN- γ stimulation, which might be responsible for LPS/IFN- γ -induced steroid insensitivity. A number of studies have shown that oxidative and nitrative stress could promote HDAC2 degradation via nitration, carbonylation, or phosphorylation (26, 27, 46). We detected prominent serine phosphorylation of HDAC2 in Raw 264.7 cells in response to LPS/IFN- γ , which might subsequently contribute to its own degradation. Activation of the PI3K/Akt signaling pathway has been well correlated with HDAC2 phosphorylation in alveolar macrophages isolated from the BAL fluid of cigarette smoke-exposed children (28). Moreover, inhibition of the PI3K/Akt pathway has been shown

FIGURE 5. Restoration of steroid sensitivity to LPS/IFN- γ -induced IL-27 and AHR by andrographolide in vivo. Lung resistance (R_L) in response to PBS control and nebulized methacholine (2.5, 5, and 10 mg/ml) was recorded and normalized over baseline. **(A)** Alteration of AHR in response to increasing doses of methacholine upon LPS, IFN- γ , and combined LPS/IFN- γ exposure in vivo. **(B)** Effect of dexamethasone and/or andrographolide on LPS/IFN- γ -induced AHR. Effects of dexamethasone and/or andrographolide on IL-27 mRNA levels **(C)** from mouse lung tissues and IL-27 protein levels **(D)** from mouse BAL fluids are shown. Data are pooled from four to seven mice. Values are expressed as mean $\pm$ SEM. ** p < 0.01, *** p < 0.001 compared with vehicle control. # p < 0.05, ## p < 0.01, ### p < 0.001 compared with PBS control.



to restore steroid sensitivity in a cigarette smoke-induced airway inflammation mouse model and in PBMCs from COPD patients via a recovery of HDAC2 level and activity (23, 27). Thus, the steroid resensitization action of andrographolide may be due to its ability to inhibit the PI3K/Akt pathway and HDAC2 phosphorylation, to restore the HDAC2 level and total HDAC activity, and to decrease the total HAT/HDAC activity ratio in alveolar macrophage and lung tissues.

Alternatively, HDAC2 and Nrf2 knockout mice exhibited steroid resistance in LPS- and cigarette smoke-induced airway inflammation, and HDAC2 protein level was found to be reduced in Nrf2 knockout mice, indicating a causative role of Nrf2 in regulating HDAC2 level (47). Interestingly, decreased HDAC2 level was also found to reduce Nrf2 stability and impair antioxidant defense (48). These findings suggest a critical interaction and interdependence of HDAC2 and Nrf2. Andrographolide has been reported to be the most robust Nrf2 inducer by screening a spectrum library of 2000 biologically active compounds using a Neh2-luciferase reporter assay (49). The present study also showed that andrographolide markedly upregulated nuclear Nrf2 levels in LPS/IFN- γ -stimulated Raw 264.7 cells and enhanced Nrf2 transcription factor binding activity to DNA in mouse lung tissues.

Taken together, this study reveals, to our knowledge for the first time, that andrographolide can resensitize the action of steroids to suppress LPS/IFN- γ -induced IL-27 production and AHR in an experimental steroid-resistant model, probably through inhibition of the PI3K/Akt/HDAC2 pathway, upregulation of Nrf2 and further restoration of HDAC2 level and total HDAC activity, and downregulation of the total HAT/HDAC activity ratio. Our data illustrate the potential therapeutic value of andrographolide in treating steroid-refractory respiratory diseases.

Disclosures

The authors have no financial conflicts of interest.

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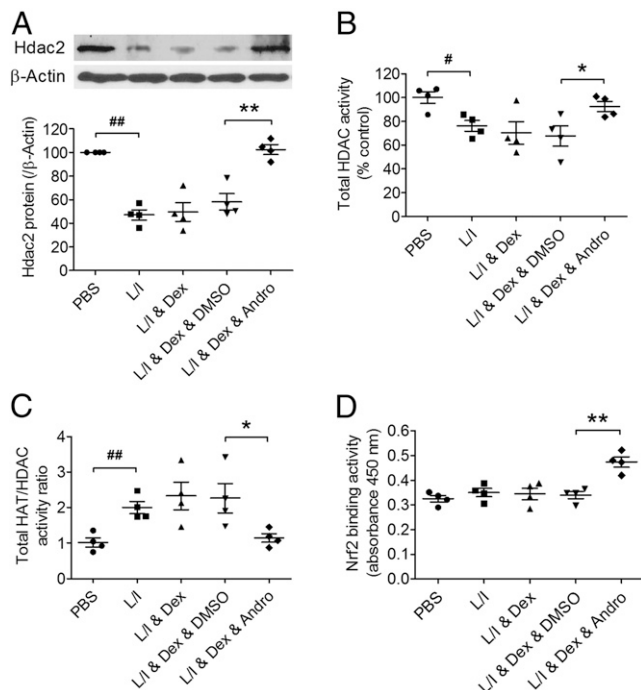


FIGURE 6. Elevation of HDAC2 protein level, total HDAC activity, and Nrf2 binding activity in mouse lung tissues. **(A)** Immunoblot and corresponding quantification of mouse lung HDAC2 protein levels in response to dexamethasone with and without andrographolide after intratracheal exposure to LPS/IFN- γ in vivo. Band intensity was quantified using ImageJ software. **(B)** Total HDAC activity and **(C)** total HAT/HDAC activity ratio in mouse lung tissues. **(D)** Nrf2 transcription factor binding activity to DNA in mouse lung tissues. Data were pooled from four to five mice. Values are expressed as mean $\pm$ SEM. * p < 0.05, ** p < 0.01 compared with vehicle control. # p < 0.05, ## p < 0.01 compared with PBS control.

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