IL-27/IFN-γ Induce MyD88-Dependent Steroid-Resistant Airway Hyperresponsiveness by Inhibiting Glucocorticoid Signaling in Macrophages

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Asthma is a chronic inflammatory disorder of the airways that is clinically characterized by recurrent airflow obstruction, wheezing, and airway hyperresponsiveness (AHR) (1). AHR is an exaggerated narrowing of the airways in response to nonspecific spasmogenic stimuli (1–3). Importantly, the degree of AHR often correlates with the severity of disease, and titration of therapy based on the control of AHR may produce superior outcomes in asthma (3). CD4+ Th2 lymphocytes have been identified as a central component of the allergic inflammatory response (4). However, there is increasing evidence that asthma is a heterogeneous inflammatory condition in which the contribution of Th2-driven pathways is not always dominant. Indeed, responsiveness to inhaled corticosteroids was recently shown to correlate with the molecular phenotype, defined in terms of the degree of Th2 inflammation (5).

In severe and difficult-to-manage forms of the disease, where steroids may have limited efficacy, factors associated with activation of host defense pathways (such as IFN-γ and LPS) are likely to contribute to pathogenesis (6–15). Patients with refractory asthma are more susceptible to allergen- and infection-induced exacerbations, and they often have increased neutrophilic rather than eosinophilic infiltrates (6, 13, 14). Although eosinophilic inflammation may still be important in severe asthma (1), the lack of control of inflammation and AHR by steroid therapy in these patients suggests that factors underpinning the expression of disease are either not controlled by glucocorticoid-responsive elements or are suppressors of this pathway.

Two factors that may be relevant in this context are the expression of IFN-γ by inflammatory cells and the activation of inflammatory cells by LPS. An increased percentage of IFN-γ-expressing cells is found in the airways of severe asthmatics, and this cytokine has been linked to the mechanisms regulating AHR (11, 16). LPS is a major component of the cell walls of Gram-negative bacteria and of allergens that trigger asthma. High levels of LPS have been detected in the bronchoalveolar lavage fluid (BALF) from steroid-resistant asthmatics and are an important determinant of the severity of asthma (9, 17). We and others have shown that IFN-γ–producing cells and LPS contribute to the induction of airway inflammation and AHR in mouse models; furthermore, we have demonstrated that IFN-γ/LPS–induced responses are steroid resistant (18, 19). Our investigations revealed a novel interaction between IFN-γ and LPS that leads to the activation of pulmonary macrophages and the induction of steroid-resistant AHR (19). Specifically, we provided the first demonstration that LPS-triggered signaling through TLR4 and MyD88 pathways cooperated with signaling through

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Inflammation and airway hyperresponsiveness (AHR) are hallmark features of asthma and often correlate with the severity of clinical disease. Although these features of asthma can be effectively managed with glucocorticoid therapy, a subgroup of patients, typically with severe asthma, remains refractory to therapy. The mechanisms leading to steroid resistance in severe asthmatics are poorly understood but may be related to the activation of innate host defense pathways. Previously, we have shown that IFN-γ–producing cells and LPS, two factors that are associated with severe asthma, induce steroid-resistant AHR in a mouse model. We now demonstrate that cooperative signaling induced by IFN-γ and LPS results in the production of IL-27 by mouse pulmonary macrophages. IL-27 and IFN-γ uniquely cooperate to induce glucocorticoid-resistant AHR through a previously unknown MyD88-dependent mechanism in pulmonary macrophages. Importantly, integrated signaling by IL-27/IFN-γ inhibits glucocorticoid-induced translocation of the glucocorticoid receptor to the nucleus of macrophages. Furthermore, expression of both IL-27 and IFN-γ was increased in the induced sputum of steroid-refractory asthmatics. These results suggest that a potential mechanism for steroid resistance in asthma is the activation of MyD88-dependent pathways in macrophages that are triggered by IL-27 and IFN-γ, and that manipulation of these pathways may be a therapeutic target.
the IFN-γR to modulate airway inflammation and reactivity to spasmogens (19). These data were consistent with earlier in vitro observations suggesting that these pathways may be integrated and regulate inflammation in macrophages (20) and block the anti-inflammatory effects of glucocorticoids in human monocytes (21).

Nevertheless, the critical downstream molecules that regulate IFN-γ and LPS–TLR4/MyD88–mediated inflammation and steroid-resistant AHR remain unknown. Analysis of recent studies suggests that IL-27 may be an important mediator of IFN-γ and LPS induced steroid-resistant AHR, because of its potential role as a regulator of pulmonary macrophage function (22–24). A recently discovered member of the IL-6/IL-12 family, IL-27 is a heterodimeric cytokine that has two subunits, an EBV-induced gene 3 (EBI3) and a p28 chain (25). It is produced by activated monocytes (or monocyte-derived dendritic cells [DCs]) and macrophages after microbial exposure (24, 25) and its expression is critically dependent on signaling through MyD88 (26, 27). The effect of IL-27 on monocytes/macrophages is complex, as it is able to induce or suppress the expression of surface activation molecules or cytokines by these cells (23, 28, 30–32). The pleiotropic effects of IL-27 on monocytes/macrophages may depend on the timing and context of surrounding inflammatory signals and the nature of the local immune environment.

The contribution of IL-27 in different phenotypes of asthma remains poorly understood. However, IL-27 is known to promote IFN-γ production by Th1 cells, as well as innate host defense responses to infection (25, 33), both of which may contribute to severe forms of asthma and exacerbations. In this study, we extend our investigations of molecular and cellular mechanisms regulating steroid-resistant AHR. Specifically, we demonstrate a critical role for IL-27, integrated with IFN-γ, in the activation of pulmonary macrophages in asthma. IL-27– and IFN-γ–induced AHR was dependent on MyD88 expression in macrophages, thus identifying a novel interaction between IL-27R, IFN-γ, and MyD88 signaling pathways in this cell. Importantly, this interaction prevented steroid-induced translocation of the glucocorticoid receptor (GR) to the nucleus of pulmonary macrophages. We also observed that the expression of IL-27 and IFN-γ was significantly greater in the sputum of patients with neutrophilic asthma, compared with those with eosinophilic asthma. Collectively, our observations suggest that a novel IL-27– and IFN-γ–driven pathway may be important in the regulation of AHR, and that the innate immune system may thus potentially contribute to the pathogenesis of steroid-resistant asthma.

Materials and Methods

Animals

Wild-type– and MyD88 knockout (MyD88−/−)-specific pathogen-free BALB/c mice (6–8 wk) were obtained from the animal services unit of the University of Newcastle. MyD88−/− mice were backcrossed for 12 generations onto the BALB/c strain. All experiments were performed with approval from the animal ethics committee of the University of Newcastle.

Administration of IL-27, IFN-γ, and/or LPS, and Ab neutralization of IL-27

Mice were anesthetized (i.v. of 100 μl Saffan solution [1:4] diluted with PBS) and the trachea was intubated with a 22-gauge catheter needle. Optimized doses of murine rIL-27 (200 ng/mouse; R&D Systems, Minneapolis, Minn), rIFN-γ (1.5 μg/mouse; PeproTech, Rocky Hill, NJ) and/or LPS (50 ng/mouse; Sigma-Aldrich, St Louis, MO), or control vehicle (0.1% BSA/PBS) were intratracheally (i.t.) instilled into the airways. Anti–IL-27 Ab (100 μg/mouse; R&D Systems) or isotype control was delivered i.v. into some mice at the same time as treatment with IFN-γ/LPS. Endpoints were assessed 12 h after treatment with IL-27, IFN-γ, and/or LPS.

Analysis of induced sputum samples from human asthma patients

Patients with asthma, defined by clinical diagnosis with evidence of AHR to hypertonic saline and/or bronchodilator response (34), were recruited and categorized via induced sputum inflammatory cell counts. Participants with a sputum eosinophil count of ≥5% in the absence of an increase in sputum neutrophil numbers were classified as eosinophilic asthma. Participants with a sputum neutrophil count of ≥63% and ≤3% eosinophils were classified as neutrophilic asthma. All participants gave written informed consent prior to their inclusion in the study, which was approved by the Hunter New England Area Health Service and the University of Newcastle Ethics Committees.

Induced sputum samples were obtained using nebulized hypertonic (45%) saline (50 ml). For the assessment of inflammatory cells, DTT was used to disperse cells from mucus. Total cell counts and cell viability (trypan blue exclusion) were performed with a hemocytometer, followed by preparation of cytopsin for differential cell counts using May-Grunwald-Giemsa. Selected sputum plugs (100 μl) were stored for RNA analysis in RLT buffer (Qiagen, Valencia, CA) at −80°C.

RNA was extracted from induced sputum plugs using RNeasy Mini kits (Qiagen) and quantitated using the Quanti-T-RiboGreen assay (Invitrogen, Carlsbad, CA). RNA (200 ng) was reverse-transcribed to cDNA using high-capacity cDNA reverse transcription kits according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). TaqMan qPCR primer and probes for IFN-γ, IL-27 p28, and EB15 genes were purchased in kit form (Applied Biosystems) and combined in duplex real-time PCR using an ABI 7500 real-time PCR machine (Applied Biosystems). The levels of expression of IFN-γ and IL-27 p28 and EB15 subunit genes were calculated using 2−ΔΔCt relative to the reference gene eukaryotic 18S RNA and an internal calibrator.

Administration of dexamethasone

Dexamethasone (DEX) (1 mg/kg; Sigma-Aldrich) was administered by daily i.p. injection for 3 consecutive days, commencing 3 d before IL-27/IFN-γ or IFN-γ/LPS treatment. Endpoints were assessed 12 h after treatment with IL-27/IFN-γ or IFN-γ/LPS.

Measurement of lung function

Airway responses to methacholine challenge were measured using Flexivent apparatus (Scireq; Montreal, Quebec, Canada) as previously reported (19). Briefly, mice were anesthetized (50 μl/10 g i.p.) with a mixture containing xylazine (2 mg/ml; Troy Laboratories, Smithfield, New South Wales, Australia) and ketamine (40 mg/ml; Parke-Davis, Australia). A further dose (20 mg/ml) of anesthetic was administered when the animal was attached to the ventilator and every 30 min thereafter. A tracheostomy was performed and a cannula (length, 1.0 cm; internal diameter, 0.0813 cm) inserted into the trachea. Animals were ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min, with a positive end-expiratory pressure of 2 cm H2O. Mice were then challenged with aerosolized saline followed by increasing concentrations of methacholine (5, 10, 20, and 40 mg/ml; Sigma-Aldrich) for 10 s at each dose. Aerosols were delivered by an ultrasonic nebulizer (Scireq) installed in a bypass branch of the inspiratory tubing. The constant-phase model was used to describe the mechanical properties of airways and parenchyma (36). Measurements were excluded if the coefficient of determination was <95%. R95% represents the Newtonian resistance of the airways, which predominantly reflects airflow, and AHR was defined as a significantly increased change in R95% relative to control mice. Changes in R95% were calculated as percentage increase over saline control.

Isolation of adherent cells, epithelial cells, and nonadherent cells

Pulmonary macrophages were isolated from mouse lungs according to previously described methods with some modifications (37). Briefly, macrophages were mechanically extracted from minced mouse lung tissue, purified by gradient centrifugation (Histopaque-1083; Sigma-Aldrich) and plated at a concentration of 1 × 106 cells/ml in RPMI 1640 containing 10% FCS. After 3 h, 95% of adherent cells were macrophages, which was confirmed by FACS. To obtain epithelial cells, lung pieces (after macrophages were mechanically extracted) were further incubated with 5 volumes of trypsin for 15 min at 37°C. Enzymatic digestion was repeated once. Cells released during the second digestion were cultured as described for airway epithelial cells (37). More than 90% of adherent cells were of the epithelial cell population, which was determined by FACS with positive staining of anti-epithelial cell adhesion molecule (mouse epithelial cell marker; BioLegend, San Diego, CA). Nonadherent cells were collected

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from macrophage and epithelial cell isolations and cultured at a concentration of 5 × 10^5 cells/ml in RPMI 1640 containing 10% FCS.

**Analysis of cytokines by ELISA**

IL-27 concentrations were determined in supernatants from IFN-γ- and/or LPS-stimulated and nonstimulated (control) macrophages, epithelial cells, and nonadherent cells isolated from mouse lung by ELISA according to the manufacturer’s instructions (R&D Systems).

**Quantitative PCR**

Quantitative PCR was performed as previously described (38). Briefly, RNA was prepared from cells or tissue using the TRIzol RNA isolation buffer following the manufacturer’s instructions (Invitrogen). cDNA was synthesized using oligo(dT)-primed reverse transcriptase reactions using 0.5 μg RNA from each sample. Quantitative PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems) using the following primers: murine IL-27 p28 (p28) (forward, 5'-CTGTGGCTGTACCTTTGGTF-3', and reverse, 5'-CACCCTCTGAACATGAGATCC-3'); EBI3 (forward, 5'-GCAACCTCTCAGCTTTTGGT-3', and reverse, 5'-GGAGCTGCTACTTGAGGAGAA-3'); and GAPDH (forward, 5'-CAAGGTCTGCTGTCG-GGCTT-3', and reverse, 5'-CCCTGTTGCTTGTAAGCCTA-3'). SYBR Green was used to detect changes in amplicon levels with each sequential amplification cycle. The fluorescence intensity was normalized to the rhodamine derivative ROX as a passive reference label, which was present in the buffer solution. The levels of mRNA from treated groups were normalized to GAPDH.

**Inhibition of NF-κB, JNK, or p38 activation**

NF-κB and JNK were depleted by i.p. injection with BAY11-7082 (10 mg/kg; Calbiochem, Darmstadt, Germany) or by i.v. injection with SP600125 (45). Briefly, phosphatidylcholine (86 mg; Sigma-Aldrich) and cholesterol (450 mg; Calbiochem, Darmstadt, Germany), or by i.v. injection with SP600125 (10 mg/kg, Sigma-Aldrich), or vehicle (2% DMSO/saline) 1 h before IFN-γ/LPS or IFN-γ/LPS treatment. Previous studies have shown that these doses of BAY11-7082 or SP600125 can efficiently inhibit the activation of NF-κB (39) and JNK (40).

For inhibition of p38, mice were i.v. injected with SB203585 (10 mg/kg; LC Laboratories, Wobum, MA) or vehicle control 1 h before IFN-γ/LPS or IFN-γ/LPS treatment. An additional dose was administered 1 h before the measurement of lung function. Previous studies have shown that these doses of SB203585 used can efficiently inhibit p38 activation (41).

**Depletion of IFN-γ, CD4+, CD8+, and NK cells and pulmonary macrophages**

CD4+ or CD8+ cells were depleted by i.p. injection with 500 μg anti-CD4 (GK1.5) (42) or 500 μg anti-CD8 (YT169.4) mAbs (43) or the corresponding isotype controls 3 and 1 h before IL-27/IFN-γ or IFN-γ/LPS treatment. Depletion of CD4+ or CD8+ cells was confirmed by FACS. NK cells were depleted by i.v. injection with 50 μl anti-ASIALO GM1 Ab (Wako Chemicals, Osaka, Japan) or rabbit serum 2 d and 1 h before IL-27/IFN-γ instillation (44). The efficiency of depletion of NK cells by anti-ASIALO GM1 Ab was determined as previously described and confirmed by FACS.

Pulmonary macrophages were depleted using liposome-encapsulated clodronate (lipo-Cl2MDP), which was prepared as described previously (45). Briefly, phosphatidylcholine (86 mg; Sigma-Aldrich) and cholesterol (8 mg; Sigma-Aldrich) were dissolved in chloroform (10 ml). A lipid film was generated by vacuum rotary evaporation and resuspended in either 4 ml PBS or 10 ml C12MDP (Sigma-Aldrich). The suspension was maintained at room temperature (RT) for 2 h, sonicated for 3 min, and again maintained for 2 h at RT. Lipo-Cl2MDP was diluted in 90 ml PBS and centrifuged at 100,000 × g for 30 min. Pellets were washed with 10 ml PBS and resuspended in 4 ml PBS. Liposomes were either used immediately or stored under N2 gas at 4°C for up to 1 wk. Either lipo-Cl2MDP or liposome-encapsulated PBS (100 μl/mouse) was i.v. instilled to deplete pulmonary macrophages 3 d before IL-27/IFN-γ or IFN-γ/LPS treatment. Cellular depletion was confirmed by examination of BALF and FACS.

**Flow cytometry**

Lungs were isolated from mice after treatment with IL-27, IFN-γ, and/or LPS. Samples were dissociated into single-cell suspensions and depleted of erythrocytes using 0.86% (w/v) ammonium chloride. Cells were then washed and immediately stained for surface marker expression using the following antibodies: anti-F4/80 (BD Pharimingen, San Diego, CA) and anti-CD11c (BD Pharimingen, San Diego, CA). Subpopulations of pulmonary macrophages were categorized as previously described (46). Briefly, typical alveolar macrophages were defined as F4/80+CD11b+CD11c+highFS−SS−high; CD11b+ macrophages as F4/80+CD11b+CD11c+lowFS−SS−; monocytes as F4/80−CD11b+CD11c+lowFS−SS−; and DCs as F4/80−CD11b+CD11c+highFS−SS−. Anti-CD3, anti-CD4, and anti-CD8 (BioLegend) were used to detect CD4+ or CD8+ T cells. Anti-CD49b and anti-FcRII (BioLegend) were used to detect NK cells (CD49b/FcRII+). All samples were analyzed using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA).

**Transfer of isolated wild-type macrophages into MyD88−/− mice**

Pulmonary macrophages from wild-type (WT) mice were isolated, harvested, and resuspended in sterile PBS. MyD88−/− mice were i.v. injected with 3 × 10^6 cells per mouse. Some cells were labeled with CFSE to confirm transfer efficiency. One hour after macrophage transfer, recipients were i.e. administered either IL-27/IFN-γ (200 ng and 1.5 μg, respectively) or vehicle.

**Immunofluorescence detection of GRs**

The detection of GRs was performed as described previously with minor modification (47). Briefly, macrophages isolated from mouse lungs were seeded on cover slips at 3 × 10^5 cells/ml and allowed to settle and adhere for 48 h before treatment at 37°C under 5% CO2. Cells were then stimulated with vehicle, LPS (50 ng/ml), IFN-γ (1.5 μg/ml), IL-27 (200 ng/ml), and/or LPS plus IFN-γ (1.5 μg/ml) plus LPS (50 ng/ml) or IL-27 (200 ng/ml) plus anti–IL-27 Ab (50 μg/ml), or IFN-γ (1.5 μg/ml) plus LPS (50 ng/ml) plus control Ab (70 μg/ml) for 24 h and treated with 1 μM DEX or vehicle for 1 h.

Pulmonary macrophages were washed with cold PBS, fixed in 4% (w/v) paraformaldehyde in PBS buffer for 20 min on ice, permeabilized with 0.2% (v/v) Triton X-100 for 10 min at RT, and blocked with 5% BSA/PBS for 30 min at RT. Cells were then incubated with the polyclonal Ab to GRs (Santa Cruz Biotechnology, Santa Cruz, CA) or control (purified nonimmune rabbit IgG [Santa Cruz Biotechnology]) diluted (1:50) in 1% BSA/PBS overnight at 4°C. Macrophages were washed with PBS for 15 min at RT and incubated with Cy3-conjugated goat anti-rabbit IgG (10 μg/ml; GE Healthcare, Buckinghamshire, U.K.) diluted in 1% BSA/CBS/PBS for 45 min in the dark at 37°C, and again washed with PBS for 15 min. The nucleus was stained with DAPI (Sigma-Aldrich) for 10 min at RT. Slides were washed with PBS and mounted. GRs in macrophages were visualized using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) with a ×100 objective lens; images were captured using a digital camera (DP70; Olympus) and analyzed using Image J 1.42 software (National Institutes of Health, Bethesda, MD). Sampling was performed on 8–10 different areas for 40–60 cells of each slide. The intensity of Cy3 staining per unit area in the nucleus and cytoplasm of each cell was quantified and the ratios of nucleus:cytoplasm were calculated.

**Data analysis**

An initial one-way ANOVA (or a Kruskal-Wallis test for nonparametric data) was followed by appropriate comparisons to test for differences between means of groups. Values are reported as the mean ± SEM for each experimental group. The number of experiments in each group ranged from 8 to 10. Differences in means were considered significant if p < 0.05.

**Results**

**IFN-γ/LPS increases activation and numbers of CD11b+ pulmonary macrophages**

We have previously shown that pulmonary macrophages have a critical role in the development of IFN-γ/LPS–induced AHR (19). This steroid-resistant AHR develops 12 h after exposure of the airways to these factors. To characterize the phenotype of these macrophages, we administered IFN-γ/LPS to the lung and 12 h later performed FACS analysis on the cells isolated from lung homogenates (see Materials and Methods for macrophage phenotypes). A greater frequency of CD11b+ macrophages (F4/80+CD11b+highCD11c+lowFS−SS−high) was observed in lungs of mice treated with IFN-γ/LPS (34.2%) compared with treatment with IFN-γ (25.4%), LPS (20.6%), or vehicle alone (20.1%) (Fig. 1A). A significant increase in the absolute numbers of CD11b+ macrophages in the lungs of IFN-γ/LPS–treated mice (2.26 ± 0.14 × 10^5 cells/ml; data expressed as mean ± SEM, n = 6, p < 0.05) was also detected compared with controls treated with IFN-γ (1.35 ± 0.23 × 10^5 cells/
IL-27/IFN-γ induce steroid-resistant AHR

In preliminary experiments, we dissected airways from lung tissue of mice 12 h after IFN-γ/LPS treatment, performed gene array analysis on these airways, and identified that IL-27 p28 was up-regulated following treatment with IFN-γ/LPS compared with vehicle treatment (unpublished data). These results support the concept that interactions between IL-27 and IFN-γ may be involved in the pathogenesis of asthma, and they are particularly relevant to the difficult-to-manage neutrophilic form of the condition.

Cooperative interaction between IL-27 and IFN-γ contributes to the development of AHR

To elucidate the role of IL-27 in the mechanism of steroid-resistant AHR, we treated mice with anti-IL-27–neutralizing Ab, exposed the airways to IFN-γ/LPS, and measured airway reactivity and inflammatory infiltrates 12 h later. Neutralization of IL-27 abolished IFN-γ/LPS–induced AHR, but not neutrophil influx, into the airways (Fig. 2A, 2B).

Next we treated mice with vehicle, IL-27, IL-27/IFN-γ, or IL-27/LPS and assessed AHR and airway inflammation 12 h later. Treatment with IL-27/IFN-γ resulted in significantly increased airway reactivity to methacholine compared with the other groups

Levels of IL-27 p28 and IFN-γ are concurrently increased in neutrophilic but not eosinophilic asthma

To determine the relevance of our observations to human disease, we measured the levels of IL-27 p28 and IFN-γ in induced sputum samples from patients with neutrophilic and eosinophilic asthma using quantitative PCR. Levels of IL-27 p28 and IFN-γ were significantly increased in neutrophilic asthma compared with eosinophilic asthma (unpublished data). These results indicate that the development of IFN-γ/LPS–induced AHR is associated with the production of IL-27 by pulmonary macrophages.

In clinical studies, the levels of CD14 on pulmonary macrophages after allergen challenge correlates with airway sensitivity to methacholine (48). To determine the effect of IFN-γ/LPS on the activation of pulmonary macrophages, we also examined the expression of CD14 on this cell population. Treatment with IFN-γ/LPS up-regulated the expression of CD14 on CD11b+ macrophages compared with controls (Fig. 1B). IFN-γ treatment alone also slightly increased CD14 expression on these cells compared with LPS or vehicle-treated controls. These data suggest that the development of IFN-γ/LPS–induced AHR is associated with activation and increased numbers of CD11b+ pulmonary macrophages.

IFN-γ/LPS administration induces increased expression of IL-27 in pulmonary macrophages

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FIGURE 1. Exposure of the airway to IFN-γ and LPS induces recruitment and activation of CD11b+ macrophages, with increased production of IL-27 from macrophages. Exposure of the airways to IFN-γ and LPS induces increases in (A) CD11b+ macrophages, (B) expression of CD14 on CD11b+ macrophages, (C) expression of IL-27 p28 mRNA in the airway tissues, and (D) production of IL-27 protein in cultured pulmonary macrophages (data are representative of four independent experiments). Levels of both (E) IL-27 p28 and IFN-γ were significantly and concurrently increased in induced sputum samples from neutrophilic (n = 9) compared with eosinophilic asthmatics (n = 11). *p < 0.05 compared with other groups; #p < 0.05 compared with groups treated with LPS, IFN-γ, or IFN-γ/LPS; •p < 0.05 compared with eosinophilic asthmatics.
IL-27/IP-γ–induced AHR is abolished by the depletion of pulmonary macrophages

To examine if pulmonary macrophages are critical in IL-27/IP-γ–induced AHR, we depleted these cells using lipo-ClsMDP 3 d before IL-27/IP-γ treatment. Depletion of pulmonary macrophages abolished IL-27/IP-γ–induced AHR (Fig. 3A). Because IL-27Rs are also found on CD4+ and CD8+ T lymphocytes and NK cells, we also tested the effect of depleting these cells with mAbs to determine their roles in IL-27/IP-γ–induced AHR. In contrast to the macrophage depletion studies, depletion of CD4+ or CD8+ T cells or NK cells did not affect IL-27/IP-γ–induced AHR (Fig. 3B–D). These data identify the critical role of pulmonary macrophages in the development of IL-27/IP-γ–induced AHR.

IL-27/IP-γ–induced AHR is not inhibited by the suppression of NF-κB, JNK, or p38

NF-κB, JNK, and p38 are critical mediators of TLR-MyD88 signaling pathways in response to LPS (49). Inhibition of NF-κB by BAY11-7082 significantly suppressed IFN-γ/LPS– but not IL-27/IP-γ–induced AHR (Fig. 4A, 4B). Activation of NF-κB is important for production of IL-27 p28 in macrophages after IFN-γ/ LPS stimulation (24). BAY11-7082 also suppressed IL-27 production from macrophages treated with IFN-γ/LPS compared with vehicle-treated controls (unpublished data). Therefore, NF-κB appears to be important in IFN-γ/LPS–induced AHR because of its role in the production of IL-27, but it is not required for IL-27/IP-γ–induced AHR. Blocking JNK (Fig. 4C, 4D) or p38 (Fig. 4E, 4F) did not affect either IFN-γ/LPS– or IL-27/IP-γ–induced AHR. Collectively, these data suggest that NF-κB, JNK, or p38 do not contribute to IL-27/IP-γ–induced AHR.

IL-27/IP-γ–induced AHR is underpinned by the activation of MyD88 in pulmonary macrophages

MyD88 has been shown in vitro to contribute to IFN-γ–regulated macrophage activation (50). To examine the role of MyD88 in IL-27/IP-γ–induced AHR, we treated mice deficient in this molecule (MyD88−/−) with IL-27/IP-γ. MyD88−/− mice did not develop AHR after administration of IL-27/IP-γ (Fig. 5A), and pulmonary macrophage numbers in BALF were the same as in WT mice (unpublished data). Notably, baseline reactivity to methacholine was equivalent in MyD88−/− and WT mice (Fig. 5A). To investigate the importance of MyD88 in macrophages in IL-27/IP-γ–induced AHR, we adoptively transferred macrophages derived from the lungs of WT mice into MyD88−/− mice and then treated these animals with IL-27/IP-γ. Transfer of WT macrophages reconstituted the IL-27/IP-γ–induced AHR in MyD88−/− mice (Fig. 5B). Baseline reactivity to methacholine in these recipient mice was also equivalent to responses in WT mice. Therefore, the presence of MyD88 in macrophages is essential for the development of IL-27/IP-γ–induced AHR, and macrophage-derived factors appear to have a central role in the induction of enhanced airway reactivity.

IL-27/IP-γ induces steroid-resistant AHR and suppresses the translocation of the GR to the nucleus of pulmonary macrophages

Next we determined if IL-27/IP-γ–induced AHR was affected by steroid treatment. Treatment of mice with DEX did not suppress AHR induced by IL-27/IP-γ (Fig. 6), indicating that IL-27 was
IFN-γ of GR (GR staining nuclear/cytoplasm ratio, 2.74 ± 0.21 or 2.25 ± 0.32, respectively; n = 4, p < 0.05) did not inhibit nuclear translocation of GR in pulmonary macrophages. Exposure of pulmonary macrophages to LPS, IFN-γ, IL-27, IFN-γ/LPS, or IL-27/IFN-γ did not affect the translocation of the GR to nucleus, whereas the receptor was largely remained in the cytoplasm in vehicle-treated controls (Fig. 4). Experiments of anti–IL-27 inhibited IFN-γ/LPS–induced blockade of GR nuclear translocation (GR staining nuclear/cytoplasm ratio, 0.76 ± 0.23 or 0.80 ± 0.32, respectively; n = 4, p < 0.05 compared with the cells treated with DEX only (as above), and levels of cytoplasmic GR were not significantly different from vehicle control in the absence of DEX treatment (GR staining nuclear/cytoplasm ratio, 0.55 ± 0.29; n = 4) (Fig. 7). Furthermore, neutralization of anti–IL-27 inhibited IFN-γ/LPS–induced blockade of GR nuclear translocation (GR staining nuclear/cytoplasm ratio, IFN-γ/LPS plus anti–IL-27 of 2.66 ± 0.78 or IFN-γ/LPS plus control Ab of 0.56 ± 0.14; n = 4; p < 0.05) (Fig. 7). These data indicate that exposure of pulmonary macrophages to IFN-γ/LPS or IL-27/IFN-γ results in defective nuclear translocation of GR to the nucleus, which underpins the mechanism of steroid-resistant AHR.

Discussion

The mechanisms predisposing to steroid insensitivity in asthma remain largely unknown. Because of the complexity of the inflammatory process in these patients, we have initiated studies to identify the potential contribution of key immune factors that are implicated in pathogenesis. Recently, we demonstrated the integrated signaling between IFN-γ and LPS predisposed to the induction of steroid-resistant AHR in a mouse model; these two factors are associated with severe asthma and exacerbations (19). In this study, we extend these observations and show that IL-27 production in macrophages plays a critical role in this mechanism of steroid resistance, by signaling with IFN-γ. Importantly, the effects of IL-27/IFN-γ are mediated via a novel MyD88-dependent pathway that suppresses nuclear translocation of the GR in pulmonary macrophages.
Asthma (48). CD11b is a component of the lung are almost doubled after allergen challenge in patients with macrophages (48, 53). Indeed, adoptive transfer of CD11b+ pulmonary macrophages (a small percentage of these CD11b+cells may also be DCs and LPS-induced recruitment of monocytes) into the airways and increased expression of IL-27 in the lung. Treatment of isolated pulmonary macrophages with IFN-γ/LPS substantially increased the expression of IL-27 p28 (but not the EBI3 subunit), and these cells were the primary source of the induced IL-27 production in the lung. This finding is analogous to the observation that CD11b+ microglia/macrophages are important sources of IL-27 during the development of inflammation associated with autoimmune encephalomyelitis (57). We showed that neutralization of IL-27 completely inhibited the development of IFN-γ/LPS-induced AHR, indicating that this molecule played a central role downstream of these signals in the induction of steroid-resistant AHR. Although IFN-γ/LPS regulated the production of IL-27 from macrophages, and this cytokine was required for the induction of AHR, it alone could not alter airway reactivity. Importantly, a second signal from IFN-γ was required in conjunction with IL-27 to induce AHR. We also found that IL-27/IFN-γ-mediated AHR was resistant to DEX therapy, further confirming that these molecules were integral to the mechanism of IL-27/LPS-induced steroid-resistant AHR. Thus, IFN-γ has two pivotal roles in the mechanism for the induction of AHR: the first in promoting LPS-induced production of IL-27 from macrophages, and the second in acting cooperatively with IL-27 to alter airway reactivity. Admistration of IL-27/IFN-γ to the airways did not induce neutrophil or eosinophil influx into the lung, and NK cells and CD4+ and CD8+ T cells were not required for the induction of AHR. However, IL-27/IFN-γ-induced AHR was attenuated by the depletion of pulmonary macrophages. Collectively, these observations demonstrate that IL-27 and pulmonary macrophages play a central role in the regulation of steroid-resistant AHR.

Although pulmonary macrophages are known to be critical in host defense against respiratory infection and are activated in the airways of asthmatics (51), their contribution to the disease process and the development of AHR is unclear. Exposure to Ags or increased level of LPS in the airways is associated with the activation of macrophages in asthma (17, 48). Interestingly, the levels of CD11b+ macrophages in the lung are almost doubled after allergen challenge in patients with asthma (48). CD11b is a component of α-chains of β2 integrins (52). Higher expression of CD11b reflects the activation of pulmonary macrophages (48, 53). Indeed, adoptive transfer of CD11b+ pulmonary macrophages induces exaggerated AHR and airway inflammation in a mouse model of asthma (54). This suggests that these cells may contribute to the pathogenesis of AHR and inflammation, particularly in relation to recurrent infections (53, 55, 56).

In our study, administration of IFN-γ/LPS to the airways resulted in the influx of increased numbers of activated CD11b+ pulmonary macrophages (a small percentage of these CD11b+cells

![FIGURE 7. IFN-γ/LPS and IL-27/IFN-γ administration impair steroid-induced nuclear translocation of GR in pulmonary macrophages. Treatment of IL-27/IFN-γ or IFN-γ/LPS led to deficient GR nuclear translocation in cultured pulmonary macrophages in response to DEX stimulation (original magnification ×1000; blue, DAPI nuclear staining; red, Cy3 GR). Furthermore, neutralization of IL-27 recovered GR nuclear translocation, which was inhibited by IFN-γ/LPS. Each panel is representative of four independent experiments. The ratio of GR staining for nucleus and cytoplasm was also defined (see text).](http://www.jimmunol.org/)

We have previously shown that TLR4-MyD88 signaling pathways are critical in the development of steroid-resistant AHR induced by IFN-γ/LPS (19). In this study, we have established that MyD88 also has a central role in IL-27/IFN-γ-induced steroid-resistant AHR. AHR induced by IL-27/IFN-γ was not suppressed in TLR4-deficient mice (unpublished data), and treatment with IL-27 and/or IFN-γ did not induce pulmonary neutrophilic influx, ruling out the possibility that the requirement for MyD88 was associated with low levels of endotoxin exposure during cytokine treatments. Moreover, adoptive transfer of WT macrophages to MyD88-deficient mice restored IL-27/IFN-γ–induced AHR. Taken together, these results suggest that MyD88, specifically expressed in macrophages, is critical for IL-27/IFN-γ signal transduction that leads to alterations in airway reactivity.

Although MyD88 was initially recognized as a key molecule in the transduction of TLR pathways, emerging in vitro evidence also reveals a role for this adaptor protein in IFN-γ signaling (50). IFN-γ stimulation of macrophages induces a novel physical association between IFN-γR and MyD88 and the formation of a signaling complex, termed a signalosome, without affecting IFN-γ-mediated AHR, further confirming that these molecules were integral to the mechanism of IL-27/LPS-induced steroid-resistant AHR.
Glucocorticoids suppress inflammation and asthma by attenuating the expression of inflammatory factors. This occurs through binding of the steroid to the GR, which results in the translocation of the GR to the nucleus and the inhibition of gene transcription (7). We found that the GR remained in the cytoplasm of macrophages instead of translocating to the nucleus after DEX treatment in the presence of IFN-γ/LPS or IL-27/IFN-γ. Exposure to IFN-γ, LPS, or IL-27 alone did not affect DEX-induced translocation of the GR. Collectively, our results demonstrate that IL-27/IFN-γ induce steroid-resistant AHR by suppressing steroid activation of the GR pathway. Furthermore, MyD88 has a critical role, directly or indirectly, in the regulation of GR signaling in macrophages in response to steroid activation, in addition to its well-described function as an adaptor molecule for TLRs. To our knowledge, this is the first demonstration of a collaborative role between IL-27R, IFN-γR, and MyD88 signaling network in the suppression of the GR pathway. The role of the endogenous glucocorticoid pathway in immune responses is to promote the expression of anti-inflammatory proteins, while downregulating the expression of proinflammatory proteins (7). Although speculative, the IL-27R/IFN-γR/MyD88 signaling system may act physiologically to suppress the GR pathway and promote the proinflammatory function of macrophages. Furthermore, chronic respiratory infection and activation of this signaling system could lead to desensitization and dysfunction of the GR pathway.

Our study indicates that the underlying inflammatory processes in patients are likely to have significant effects on the pattern of AHR and its responsiveness to steroid treatment. Clinical and experimental studies suggest that Th2 cell/eosinophil–dominated asthma, which is dependent on signaling through STAT6, can be effectively managed by steroids (58–61). In contrast, pathways activated by host defense mechanisms (classically nonallergic) such as IL-27/IFN-γ and Th17 cells are more likely to be steroid-resistant (12, 19, 21, 62). Thus, nonallergic pathways that lead to AHR are fundamentally different from those regulated by Ag-reactive CD4+ Th2 cells. Interestingly, nonallergic pathways may be able to cross-regulate allergic inflammatory mechanisms suppressing Th2-like disease. While IL-27 and its receptor WSX-1 are important in the initial stages of CD4+ Th1 cell differentiation (25), they also negatively regulate Th2 cell development and activity (63, 64). WSX-1–deficient mice exhibit enhanced Th2 responses in models of allergic airways inflammation and gastrointestinal nematode *Trichuris muris* infection (63, 65). Intranasal administration of IL-27 during Ag challenge in a mouse model of allergic asthma inhibited IL-13 production from Th2 cells and the development of AHR (64).

Although preliminary, analysis of the expression of IL-27 and IFN-γ in the sputum from neutrophilic and eosinophilic asthmatics supports a potential role for these factors in disease pathogenesis. Of note, IL-27 and IFN-γ were expressed to a greater extent in the airway secretions of neutrophilic asthmatics, who show insensitivity to steroid treatment. Neutrophilic airway inflammation is a recognized feature of severe and difficult-to-manage asthma, but the contribution of this cell type to the expression of disease remains unresolved. Interestingly, neutrophilic asthmatics are allergic individuals, as evidenced by positive atopic tests, yet they have substantially reduced eosinophil levels (∼0.4%) in their sputum compared with eosinophilic asthmatics (∼4.3%) (66).

In summary, the pathogenesis of severe and steroid-resistant asthma is complex, and our lack of knowledge prevents the development of targeted therapies. By focusing on the role of innate host defense pathways, we have developed models that allow dissection of the mechanisms predisposing to the induction of nonallergic inflammation and steroid-resistant AHR. In this study, we demonstrate the importance of integrated signaling events between IL-27R, IFN-γR, and MyD88 pathways specifically in pulmonary macrophages for the induction of steroid-resistant AHR. This mechanism, which does not require eosinophilic or neutrophilic inflammation and occurs independently of T lymphocytes, may be clinically relevant. Importantly, the IL-27R–linked IFN-γR pathway operates by inhibiting steroid-induced GR trafficking into the nucleus. Understanding the contribution of this novel macrophage pathway to subtypes of asthma may provide new therapeutic approaches for difficult–to-treat asthma.

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**Disclosures**

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**References**
