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

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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 Journal of Immunology, 2010, 185: 000–000.

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...
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-induced steroid-resistant AHR remain unknown. Analysis of recent studies suggests that IL-27 may be an important mediator of IFN-γ-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 (24). IL-27 acts on a wide range of immune cells, including CD4+ and CD8+ T lymphocytes, NK cells, monocytes/macrophages, and activated DCs (23, 26–29). 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-γR, 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), 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 intratracheal (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 ≥5% 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 Research Ethics Committees.

Induced sputum samples were obtained using nebulized hypertonic saline (5%) 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 cytopsins for differential cell counts using May-Grünwald-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 QNeasy Mini kits (Qiagen) and quantitated using the QuantiT-RT 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 EBI3 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 EBI3 subunit genes were calculated using 2–ΔΔCt relative to the reference gene eukaryotic 18S rRNA 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; Parnell, Alexandria, New South Wales, Australia). A further dose (20 μl/10 g) 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 volume of 2 cm H2O. Mice were then challenged with aerosolized saline for 60 s followed by incremental increases of concentration from 10, 20, 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%.

Raw represents the Newtonian resistance of the airways, which predominantly reflects airflow, and AHR was defined as a significantly increased change in Raw relative to control mice. Changes in Raw 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 resuspended 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 macrophage extraction) 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 (forward, 5'-CTGTGCTGACCTCCTGTT-3', and reverse, 5'-CAGCTTCTGAACTGATTAG-3'), and GAPDH (forward, 5'-GACGCTCTTCAAGCTTTGGT-3', and reverse, 5'-GGAGTGGCTTACTTGAGAG-3'). SYBR Green was used to detect changes in amplitude 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 (25 mg/kg; Sigma-Aldrich), or vehicle (2% DMSO/saline) 1 h before IFN-γ/LPS or IL-27/IFN-γ 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, Woburn, MA) or vehicle control 1 h before IFN-γ/LPS or IL-27/IFN-γ 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 (YTS169.4) 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-ASIALOGM1 Ab (Wako Chemicals, Osaka, Japan) or rabbit serum 2 d and 1 h before IL-27/IFN-γ installation (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 Cl2MDP (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.p. 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 fluorescent antibodies: anti-F4/80 (clone 5B10, BD PharMingen, San Diego, CA), anti-CD11c (clone 18B1, BD PharMingen, San Diego, CA), and anti-CD86 (clone GL1, BD PharMingen, San Diego, CA). Subpopulations of pulmonary macrophages were categorized as previously described (46). Briefly, typical alveolar macrophages were defined as F4/80highCD11b−CD11c−/lowFSαhighSS−high, CD11b+ macrophages as F4/80−/CD11b+CD11c−/lowFSα−/SS−high, monocytes as F4/80highCD11b+CD11c−/lowFSα−/SS−high, and DCs as F4/80−/CD11b−/CD11c+FSα−/SS−high. Anti-CD3, anti-CD4, and anti-CD8 (BioLegend) were used to detect CD4+ or CD8+ T cells. Anti-CD49b and anti-FcεRI (BioLegend) were used to detect NK cells (CD49b+FcεRI+). All samples were analyzed using a FACS Canto II 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 × 10^6 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), IL-27 (1.5 μg/ml) plus LPS (50 ng/ml), IL-27 (200 ng/ml) plus IFN-γ (1.5 μg/ml), IFN-γ (1.5 μg/ml) plus LPS (50 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/PBS for 45 min in the dark at 37°C, and again washed with PBS. Nuclei were 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 mice 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 phenotype). A greater frequency of CD11b+ macrophages (F4/80−CD11b+ high CD11c−/low) 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 × 10^7 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 × 10^7 cells/
Regulated compared with IFN-γ analysis on these airways, and identified that IL-27 p28 was up-regulated during IFN-γ treatment alone slightly increased CD14 expression compared with LPS or vehicle-treated controls (Fig. 1A). There were no significant changes in numbers of monocytes or DCs (unpublished data).

In clinical studies, the levels of CD14 on pulmonary macrophages after allergen challenge correlates with airflow 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

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 compared with IFN-γ-, LPS-, or vehicle-treated controls (unpublished data). This result was confirmed by quantitative PCR (Fig. 1C). The IL-27 EBI3 subunit was significantly increased in response to IFN-γ/LPS, IFN-γ, and LPS administration compared with vehicle-treated controls (Fig. 1C). To analyze the cellular production of IL-27 p28 protein, we separated homogenized lung into fractions highly enriched for macrophages or epithelial cells, or containing other nonadherent cells. IFN-γ/LPS treatment induced the production of IL-27 p28 by pulmonary macrophages, which was significantly increased at 6 h and peaked at 12 h (Fig. 1D). Although IFN-γ had no effect, LPS caused a slight increase in production of IL-27 p28. We did not detect production of IL-27 p28 by epithelial cells or in the nonadherent cell population (containing CD4+ and CD8+ T lymphocytes and NK cells; confirmed by FACS; unpublished data). These data indicate that the development of IFN-γ/LPS–induced AHR is associated with the production of IL-27 by pulmonary macrophages.

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 (Fig. 1E). No difference was observed in the expression of the IL-27 EBI3 between the different groups (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.
vehicle-treated controls (unpublished data). Therefore, NF-κB production from macrophages treated with IFN-γ/LPS stimulation (24). BAY11-7082 also suppressed IL-27 production, which is important for production of IL-27 p28 in macrophages after IFN-γ/LPS stimulation. BAY11-7082 significantly suppressed IFN-γ/LPS-induced NF-κB signaling pathways in response to LPS (49). Inhibition of 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/IFN-γ–induced AHR. Blocking JNK (Fig. 4C, 4D) or p38 (Fig. 4E, 4F) did not affect either IFN-γ/LPS– or IL-27/IFN-γ–induced AHR. Collectively, these data suggest that NF-κB, JNK, or p38 do not contribute to IL-27/IFN-γ–induced AHR.

IL-27/IFN-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/IFN-γ–induced AHR, we treated mice deficient in this molecule (MyD88−/−) with IL-27/IFN-γ. MyD88−/− mice did not develop AHR after administration of IL-27/IFN-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/IFN-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/IFN-γ. Transfer of WT macrophages reconstituted the IL-27/IFN-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/IFN-γ–induced AHR, and macrophage-derived factors appear to have a central role in the induction of enhanced airway reactivity.

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

Next we determined if IL-27/IFN-γ–induced AHR was affected by steroid treatment. Treatment of mice with DEX did not suppress AHR induced by IL-27/IFN-γ (Fig. 6), indicating that IL-27 was...
FIGURE 4. The development of IL-27/IFN-γ-induced AHR does not involve NF-κB, JNK, or p38. Inhibition of activation of NF-κB by administration of BAY11-7083 markedly suppressed (A) IFN-γ/LPS but did not affect (B) IL-27/IFN-γ-induced AHR. Blocking of JNK function by treatment with SP600125 did not affect (C) IFN-γ/LPS or (D) IL-27/IFN-γ-induced AHR. Inhibition of p38 action by administration of SB203585 did not affect (E) IFN-γ/LPS– or (F) IL-27/IFN-γ-induced AHR. *p < 0.05 compared with other groups; †p < 0.05 compared with vehicle controls.

FIGURE 5. The induction of AHR by IL-27/IFN-γ is dependent on MyD88 signaling in pulmonary macrophages. The instillation of IL-27/IFN-γ into the airways of (A) MyD88−/− mice did not induce AHR. However, (B) the development of IL-27/IFN-γ–induced AHR was reinstated in MyD88−/− mice by transfer of WT pulmonary macrophages. #p < 0.05 compared with other groups; †p < 0.05 compared with vehicle controls.

FIGURE 6. IFN-γ/LPS and IL-27/IFN-γ administration leads to the development of DEX-resistant AHR. IFN-γ/LPS– or IL-27/IFN-γ–induced AHR was not inhibited by DEX. ‡p < 0.05 compared with vehicle controls.

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.

integral to the steroid-resistant mechanism induced downstream in IFN-γ/LPS signaling pathways. Glucocorticoids act by binding to GR, which leads to the translocation of the resulting complex to the nucleus and the inhibition of gene transcription (7). To determine if IL-27/IFN-γ treatment altered the ability of DEX to promote the translocation of GR, we visualized its intracellular localization by immunocytochemistry in pulmonary macrophages exposed to DEX and stimulated with LPS, IFN-γ, IL-27, IFN-γ/LPS, or IL-27/IFN-γ. Exposure of macrophages to DEX promoted the translocation of the GR to nucleus, whereas the receptor largely remained in the cytoplasm in vehicle-treated controls (Fig. 7). Exposure of pulmonary macrophages to LPS, IFN-γ, or IL-27 prior to treatment with DEX did not inhibit nuclear translocation of GR (GR staining nuclear/cytoplasm ratio, 2.74 ± 1.01, 2.17 ± 0.61, or 2.25 ± 0.51, respectively; n = 4) and translocation was similar to that observed after DEX treatment alone (GR staining nuclear/cytoplasm ratio, 2.90 ± 0.81; n = 4) (Fig. 7). In contrast, IFN-γ/LPS or IL-27/IFN-γ exposure resulted in a significant reduc-
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 IFN-γ/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. Administration 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.

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 JAK-STAT signaling/phosphorylation (20). During this process, MyD88 directly interacts with the cytoplasmic domain of the IFN-γR (20). The signalosome may function to regulate specific aspects of host defense responses, as subsets of proinflammatory molecules are not transcribed in macrophages deficient in MyD88 when stimulated with IFN-γ (20). Importantly, the specific and critical role of MyD88 in IFN-γ signaling pathways was further supported in our studies by the lack of effect of the inhibition of NF-κB or other MAPK pathways (e.g., JNK and p38) on IL-27/IFN-γ–induced AHR. These molecules are important components in TLR-MyD88–linked signaling cascades. Collectively, our data indicate that the induction of IL-27/IFN-γ–induced AHR is dependent on an integrated cytokine signaling network in macrophages that involves the IL-27R, IFN-γR, and MyD88.
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 GR-positive asthmatics, yet they have substantially reduced eosinophil levels (~0.4%) in their sputum compared with eosinophilic asthmatics (~4%) (66).

In summary, the pathogenesis of severe and steroid-resistant asthma is complex, and our lack of knowledge prevents the development of targeted therapeutics. 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


