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Identification of IFN- γ and IL-27 as Critical Regulators of Respiratory Syncytial Virus–Induced Exacerbation of Allergic Airways Disease in a Mouse Model

Thi Hiep Nguyen, Steven Maltby, Hock L. Tay, Fiona Eyers, Paul S. Foster,¹ and Ming Yang¹

Respiratory syncytial virus (RSV) infection induces asthma exacerbations, which leads to worsening of clinical symptoms and may result in a sustained decline in lung function. Exacerbations are the main cause of morbidity and mortality associated with asthma, and significantly contribute to asthma-associated healthcare costs. Although glucocorticoids are used to manage exacerbations, some patients respond to them poorly. The underlying mechanisms associated with steroid-resistant exacerbations remain largely unknown. We have previously established a mouse model of RSV-induced exacerbation of allergic airways disease, which mimics hallmark clinical features of asthma. In this study, we have identified key roles for macrophage IFN- γ and IL-27 in the regulation of RSV-induced exacerbation of allergic airways disease. Production of IFN- γ and IL-27 was steroid-resistant, and neutralization of IFN- γ or IL-27 significantly suppressed RSV-induced steroid-resistant airway hyperresponsiveness and airway inflammation. We have previously implicated activation of pulmonary macrophage by TNF- α and/or MCP-1 in the mechanisms of RSV-induced exacerbation. Stimulation of pulmonary macrophages with TNF- α and/or MCP-1 induced expression of both IFN- γ and IL-27. Our findings highlight critical roles for IFN- γ and IL-27, downstream of TNF- α and MCP-1, in the mechanism of RSV-induced exacerbation. Thus, targeting the pathways that these factors activate may be a potential therapeutic approach for virus-induced asthma exacerbations. *The Journal of Immunology*, 2018, 200: 000–000.

Respiratory syncytial virus (RSV) is a single-strand negative sense RNA virus of the *Paramyxoviridae* family that causes lower respiratory tract infections and bronchiolitis in children (1, 2) and contributes to the hospitalization of immunocompromised elderly adults (3–5). RSV infection has also been found to increase susceptibility to development of asthma in later life (6–9) and is an important trigger (with rhinovirus and parainfluenza viruses) of exacerbations (10–15). Asthma exacerbations are not only acutely debilitating, but also lead to a long-term decline in lung function (16), increase the risk for future exacerbations (17), and account for the majority of healthcare costs that are associated with the management of this disorder (18, 19). Although gluco-

corticoids therapy effectively controls mild-to-moderate asthma, it often fails to improve symptoms in patients with asthma exacerbations (20, 21). Furthermore, glucocorticoids treatment (e.g., dexamethasone [Dex]) shows no beneficial effect for the treatment of children with RSV-induced bronchiolitis (22–28). Thus, understanding the mechanisms underpinning the RSV-induced exacerbation of asthma and the development of steroid resistance is central in developing new therapeutic approaches.

Recently, we have developed a mouse model of steroid-resistant RSV-induced exacerbation on the background of pre-existing allergic airways disease (AAD) (29) to gain mechanistic insights into the pathophysiological processes regulating exacerbations of asthma. With this model we have demonstrated that RSV infection leads to steroid-resistant and macrophage-driven exacerbation of airway hyperresponsiveness (AHR) and airway inflammation (29). In addition, we identified that TNF- α and MCP-1 critically regulate the function of pulmonary macrophages to induce exacerbation; however, the factors downstream of macrophage activation were not elucidated.

Respiratory infections that lead to abnormal innate immune responses and macrophage activation are emerging as important factors in the development of asthma and steroid-resistant exacerbations of the disease (30–34). For example, viral infections are strongly linked to exacerbations in association with impaired innate antiviral immunity (35–37). It is well-known that the expression of IFN- γ is significantly increased in sputum cells of patients during virus-induced exacerbations (36) and in airways of patients with severe asthma, who often respond poorly to glucocorticoid treatment (38). In addition, the levels of IL-27 are increased in bronchoalveolar lavage fluid (BALF) cells of severe asthmatic patients, compared with that of mild-to-moderate asthmatics (39). Indeed, our previous animal studies have demonstrated that the delivery of LPS and IFN- γ , two factors linked to

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; 2-CA, 2-chloroadenosine; Dex, dexamethasone; OVA/RSV, RSV inoculation of OVA-sensitized/challenged mice; PAS, periodic acid–Schiff; RSV, respiratory syncytial virus.

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steroid-resistant asthma and its severity, collectively (but not alone) to the lung induces the production of IL-27, the activation of neutrophils and macrophages, and AHR (40, 41). In this model, IFN- γ and IL-27 synergistically act to induce macrophage-dependent steroid-resistant AHR and inflammation (40). IFN- γ is produced by a wide range of immune cells including Th1 cells, macrophages, and NK cells in response to allergen or infection, and plays a crucial role in host defense (42–45). IL-27, a member of the IL6/IL-12 cytokine family, is a heterodimeric cytokine with two subunits, EBV-induced gene 3 and p28 (46). IL-27 can be produced by activated monocytes/macrophages after microbial exposure and regulate the function of a wide range of immune cells including lymphocytes, NK cells, monocytes/macrophages, and activated dendritic cells (43, 46–48). We have shown that high levels of IL-27p28 subunit (the functional subunit of IL-27), but not EBV-induced gene 3 subunit, are expressed in the sputum of neutrophilic asthmatics, who often poorly respond to glucocorticoid treatment. These clinical observations in conjunction with our animal model studies, and subsequently those of others, strongly suggest that IFN- γ and IL-27 may underpin the development of steroid-resistant mechanisms in asthma (40, 41, 49–51). However, the precise roles of IFN- γ and IL-27 in virus-induced exacerbations of AHR and airway inflammation remain unknown.

In this study, we have extended our mechanistic investigations into the pathogenesis of RSV-induced steroid-resistant exacerbation of AAD by investigating the potential roles of IFN- γ and IL-27. We observed that IFN- γ and IL-27 levels were increased during RSV-induced exacerbation, and their levels were not affected by Dex treatment. Neutralizing IFN- γ or IL-27 effectively suppressed RSV-induced steroid-resistant AHR. Further, stimulation of pulmonary macrophages with TNF- α and/or MCP-1 could induce IFN- γ and IL-27 production, suggesting a mechanistic link with our previous studies. Our findings provide further insight into the potential mechanisms underlying virus-induced asthma exacerbations and further highlight the role of the innate immune system in driving steroid-resistant pathways and IFN- γ and IL-27 as potential therapeutic targets for treatment of asthma exacerbations.

Materials and Methods

Mice

Specific pathogen-free BALB/c male mice (6–8 wk old) were obtained from the University of Newcastle Animal Services Unit, and experiments were performed in the Hunter Medical Research Institute animal facility. All protocols were approved by the Animal Care and Ethics Committee of the University of Newcastle, Australia (Ethics no. A-2013-337).

RSV production

Human RSV (long strain, type A) was propagated in Hep-2 cells as previously described (29). Supernatant and cells were collected and centrifuged to produce clarified supernatant. Virus was then concentrated by ultracentrifugation of supernatant on a two-step sucrose gradient. RSV titer was quantified by plaque assay in Hep-2 cells (52).

Experimental model

As previously described (29), mice were sensitized to OVA (50 μ g per mouse) or exposed to PBS (as nonsensitized control) in 1 mg Alhydrogel (alum) in sterile PBS on day 0 by i.p. injection. Mice were aerosol challenged with 1% OVA for 30 min/d from days 13 to 16. Where indicated, mice were intranasally inoculated with RSV (0.5×10^6 PFUs per mouse) on day 19. All endpoints were performed on day 24.

Where indicated, mice were treated with Dex (1 mg/kg; Sigma-Aldrich) by i.p. injection on days 18 and 20. For intervention studies, mice were treated by i.p. administration of 200 μ g per mouse of anti-IFN- γ mAb (clone R4-6A2; Bio X Cell), 50 μ g per mouse of anti-IL-27p28 polyclonal Ab (R&D Systems), or the isotype control Abs (rat IgG2a [Bio X Cell] or goat IgG [R&D Systems], respectively) in 200 μ l of PBS on days 20 and 22. In some studies, mice were treated by i.p. administration of 0.2 mg of anti-TNF- α

mAb (clone XT3.11; Bio X Cell) or anti-MCP-1 mAb (clone 2H5; Bio X Cell) in 200 μ l of PBS or the isotype control Abs (rat IgG2a or hamster IgG [Bio X Cell], respectively) on days 20 and 22. To deplete macrophages, we treated mice with 2-chloroadenosine (2-CA) (10 μ l of 10 mM 2-CA or PBS control) by intratracheal administration on days 20 and 22.

Measurement of lung function

Airway resistance in response to indicated concentrations of methacholine (Sigma-Aldrich) was measured using a flexiVent apparatus (FX1 system; Scireq, Montreal, QC, Canada), as previously described (29). In brief, mice were anesthetized with a mixture containing xylazine (10 mg/kg) and ketamine (100 mg/kg) by i.p. injection (ratio 1:4, respectively; 150 μ l per mouse). A cannula was then inserted into the trachea, and mice were ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min. Mice were initially challenged with aerosol saline followed by increasing concentrations of methacholine (0.3, 1, 3, 10, and 30 mg/ml in saline). Airway resistance was presented as percentage increase over baseline (saline challenge).

BALF cell counts

BALF was collected and processed as previously described (29). In brief, the left lung lobe was tied off, and the right lung lobes were washed with 0.7 ml of sterile HBSS twice. RBCs were removed by using hypotonic RBC lysis buffer, and BALF was then centrifuged to collect cellular infiltrate. Total cell numbers were quantified using a hemocytometer, and cells were cytospun onto glass slides (Thermo Fisher Scientific, Scoresby, VIC, Australia). Differential leukocyte counts were determined based on morphological criteria by light microscopy ($\times 100$) on May-Grunwald and Giemsa-stained slides, counting a minimum of 300 cells per slide per sample (53).

Pulmonary macrophage isolation and stimulation

Pulmonary macrophages were isolated from mouse lung and characterized as previously described (29). In brief, mouse lung tissues were initially forced through 70- μ m cell strainers, subjected to gradient centrifugation (Histopaque 1083; Sigma-Aldrich), and then seeded at a concentration of 1×10^6 cells/ml in DMEM containing 20% FCS. After 3 h, >95% of adherent cells were macrophages. Isolated macrophages from treated mice were collected and placed into TRIzol and then stored at -80°C until further analysis. Pulmonary macrophages were isolated from naive mice and then stimulated with indicated concentrations of TNF- α and/or MCP-1 for 12 h in vitro. After 12 h of stimulation, cell supernatants were collected and stored at -80°C for ELISA assessments later, and cells were harvested in TRIzol for mRNA quantification.

Lung histology

Lung lobes from treated mice were removed, fixed in 10% buffered formalin, embedded, and sectioned (4–6 μ m). Sections were then stained with chromotrope-hematoxylin or periodic acid–Schiff (PAS). Scorings for histopathology (inflammatory infiltrates) and PAS (mucus-producing cells) were performed according to a set of morphological criteria, as previously described (54–56). All scoring was performed blinded.

Intracellular staining

Single-cell suspensions from lungs were prepared by digesting with HEPES buffer containing collagenase D (Sigma-Aldrich) and DNase, then gently forced through a 70- μ m cell strainer. RBC lysis was performed using ammonium chloride solution. Cell suspensions were stimulated with ionomycin (1 μ g/ml) and PMA (50 ng/ml) for 1 h and incubated with brefeldin A for 4 h to block the Golgi apparatus. After staining with cell surface marker Abs, cells were fixed using 4% PFA and permeabilized using 0.2% saponin in FACS buffer. Cells were then stained with anti-IFN- γ and anti-IL-27p28 Abs. The following Abs were used (BD Biosciences): CD11b (M1/70), F4/80 (T45-2342), IFN- γ (XMG1.2), and IL-27p28 (MM27.7B1). Lung macrophages were defined as F4/80 $^{+}$ CD11b $^{+}$ FS $^{\text{high}}$ SS $^{\text{high}}$, as previously reported (40). Data acquisition was done using a FACSCanto II flow cytometer machine (BD Pharmingen), then analyzed using FACSDiva software (BD Biosciences).

RNA extraction, RT-PCR, and quantitative PCR

As previously described (29), total RNA was extracted from lung tissue or isolated macrophages using TRIzol reagent (Invitrogen) and phenol-chloroform extraction, and quantified on a NanoDrop 1000 spectrophotometer (NanoDrop). cDNA was synthesized by RT-PCR using random hexamer primers (Invitrogen) on a T100 thermal cycler (Bio-Rad).

Quantitative PCR was performed on a Viia7 real-time PCR machine (Life Technologies) using SYBR reagents with thermal cycling conditions and primers as described in previous studies (29, 40, 57, 58). Resulting mRNA levels were normalized to hypoxanthine phosphoribosyl transferase (HPRT, internal control) and expressed as a fold-change relative to control samples (PBS group). Absolute quantification of RSV levels was determined using plasmid copy number standards for the RSV N gene and HPRT.

ELISA

Lung tissue was homogenized using radioimmunoprecipitation assay buffer (Sigma-Aldrich) plus protease/phosphatase inhibitor mixture (Cell Signaling Technology, Danvers, MA) on a TissueLyser LT tissue disruptor (Qiagen, Valencia, CA) and stored at -80°C for subsequent cytokine measurement. Total protein levels in lung tissue were measured using Pierce BCA protein assay kit (Thermo Scientific). ELISA kits were used to quantify IFN- γ , IL-27p28, TNF- α , and MCP-1 according to the manufacturer's instructions (R&D Systems). Data were then normalized to total lung protein levels, which were then expressed as picograms per milligram of total lung protein.

The levels of IFN- γ and IL-27p28 in the culture supernatants of TNF- α - and/or MCP-1-stimulated pulmonary macrophage were measured using ELISA kits (eBioscience and R&D Systems, respectively), according to the manufacturer's protocol.

Statistical analysis

All data were analyzed using GraphPad Prism Software (Version 6; San Diego, CA) and presented as mean $\pm$ SEM. Lung function data were analyzed by two-way ANOVA followed by a Bonferroni post hoc test for comparison between groups. Other data were analyzed using unpaired two-way Student *t* tests. Differences were considered statistically significant for $p < 0.05$.

Results

The levels of IFN- γ and IL-27p28 are increased in lung tissue and pulmonary macrophages after RSV-induced steroid-resistant exacerbation

We have previously shown that RSV induces exacerbation of AAD, which is characterized by steroid-resistant prolonged AHR and airway inflammation (29). In this study, we have observed similar observations. Sensitization and aeroallergen challenge of mice with (OVA) induced AAD characterized by an increase in AHR (Fig. 1A), total BALF cell number (Fig. 1B) (an eosinophil-dominant cellular infiltrate; data not shown), and the levels of inflammatory infiltrates and mucus secretion (Fig. 1C), by contrast

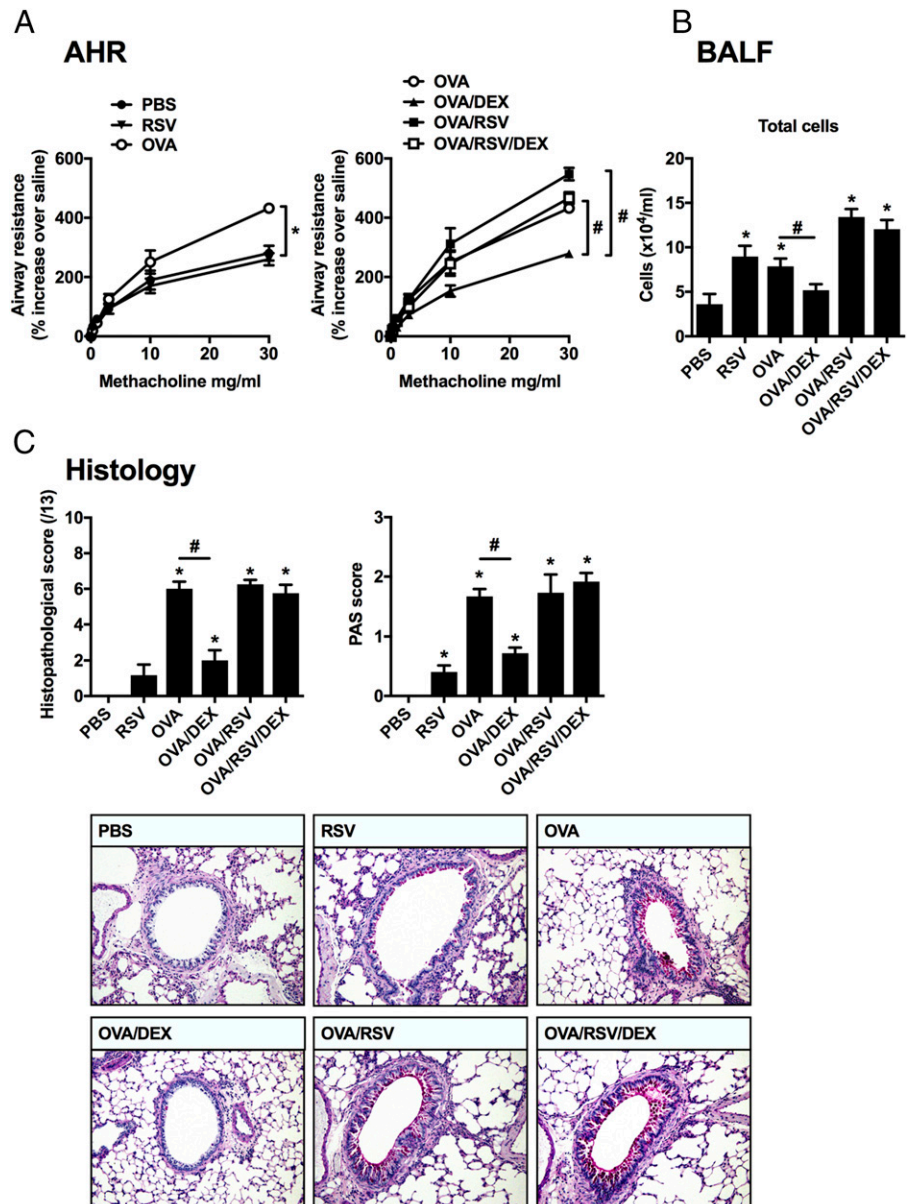
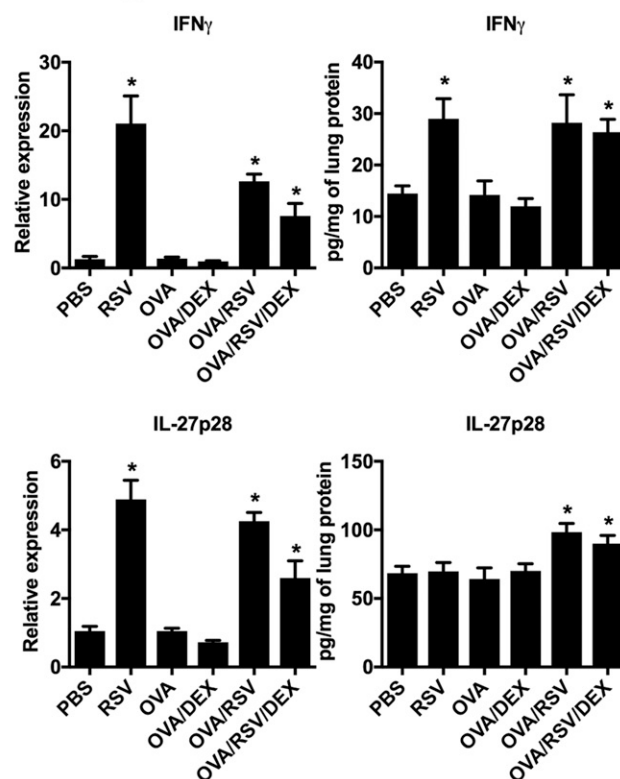


FIGURE 1. RSV infection induced AHR and increased total inflammatory cells in BALF in mice with AAD. BALB/c mice were sensitized i.p. with OVA or PBS on day 0 and exposed to OVA aerosol on days 13–16. Some mice were treated with Dex on days 18 and 20, and inoculated with RSV on day 19. AHR, total BALF cells in BALF, and histological changes were assessed on day 24. (A) AHR, (B) BALF, (C) inflammatory infiltrates, and mucus-producing cells in lung. PAS stained. Original magnification $\times 10$. Data are presented as mean $\pm$ SEM ($n = 6$ –8 mice per group). Results are representative of three independent experiments. * $p < 0.05$ designates significant differences from PBS-treated controls, # $p < 0.05$ designates significant differences between respective groups.

with PBS-treated controls (PBS group). These physiological and inflammatory responses were sensitive to Dex treatment (Fig. 1A–C) (29). RSV infection alone (RSV group) did not induce AHR compared with PBS group (Fig. 1B). However, RSV inoculation of OVA-sensitized/challenged mice (OVA/RSV) induced exaggerated AHR and total inflammatory cell recruitment compared with the noninfected OVA group (Fig. 1A, 1B). Although Dex treatment suppressed total inflammatory cell numbers in BALF and AHR in OVA-treated mice (OVA/Dex) to almost baseline levels, it did not significantly attenuate the exaggerated effects of RSV inoculation in the OVA/RSV/Dex group (Fig. 1A, 1B). Although the lungs of OVA-, OVA/RSV-, and OVA/RSV/Dex-treated mice demonstrate heightened airway inflammation and mucus production as compared with those of PBS-, RSV-, and OVA/Dex-treated mice, there was no significant difference in the histopathological scores and PAS scores between OVA, OVA/RSV, and OVA/RSV/Dex groups (Fig. 1C). Furthermore, we have also measured the levels of IL-4, IL-5, IL-13, and IL-17A in lung. The levels of IL-5 and IL-13 were unchanged in OVA-treated mice following inoculation with RSV (data not shown), as demonstrated previously (29). Expression of IL-4 increased in the lung of RSV-, OVA-, and OVA/RSV-treated mice compared with that of PBS-treated mice (Supplemental Fig. 1A). Significant increases of IL-17A following RSV, OVA/RSV, or OVA/RSV/Dex treatment were detected, as compared with treatment with PBS, OVA, or OVA/Dex (Supplemental Fig. 1B). In addition, Dex administration did not affect the production of IL-4 and IL-17A following OVA/RSV treatment. RSV infection induced high protein levels of IFN- γ , but not IL-27p28, in the RSV group compared with the PBS group (Fig. 2A). OVA/RSV treatment resulted in significantly high levels of IFN- γ and IL-27p28 transcripts and proteins in lung tissues, when compared with those of PBS, OVA, and OVA/Dex treatments (Fig. 2A). Expression and protein levels of IFN- γ and IL-27p28 were not significantly increased in the OVA treatment group compared with the PBS treatment group (Fig. 2A). Furthermore, only mRNA, but not protein, levels of IFN- γ and IL-27p28 in lung tissue were reduced by Dex treatment (OVA/RSV/Dex), compared with the OVA/RSV treatment group (Fig. 2A). Exposure of mice to UV-inactivated RSV does not induce exaggerated inflammation and AHR or alterations in cytokine levels (data not shown) (29).

We have also demonstrated that pulmonary macrophages play a critical role in the induction of AHR in our model of RSV-induced exacerbation of AAD (29). Therefore, to determine the levels of IFN- γ and IL-27p28 produced by these cells, we isolated pulmonary macrophages from the lung of treated mice (Fig. 2B). Macrophages isolated from OVA/RSV-treated mice expressed significantly higher levels of IFN- γ and IL-27p28, compared with those cells from PBS- or OVA-treated mice (Fig. 2B). Although macrophages isolated from RSV-treated mice produced high levels of both IFN- γ and IL-27p28 compared with PBS controls, the levels of IFN- γ in those cells from RSV-treated mice were significantly lower than those from OVA/RSV-treated mice (Fig. 2B). Notably, the increased expression of IFN- γ and IL-27p28 by OVA/RSV treatment was not affected by Dex administration (OVA/RSV/Dex) (Fig. 2B). In addition, OVA or OVA/Dex treatments did not influence the expression of these two cytokines (Fig. 2B). Furthermore, we have observed that a significant number of lung macrophages in OVA/RSV- and OVA/RSV/Dex-treated mice produce IFN- γ or IL-27p28 by intracellular staining, at $2.5 \pm 0.8 \times 10^4$ or $3.5 \pm 0.7 \times 10^4$ cells/ml, respectively (Supplemental Fig. 2). Interestingly, albeit at a very low level, a small number ($0.25 \pm 0.07 \times 10^4$ cells/ml) of F4/80⁺CD11b⁺ lung macrophages produced both IFN- γ and IL-27 in response to OVA/RSV treatment (Supplemental Fig. 2D, 2E). The numbers of F4/80⁺CD11b⁺ lung macrophages producing IFN- γ and/or IL-27 were not affected by Dex administration. Our data demonstrate

A Lung tissue



B Pulmonary macrophages

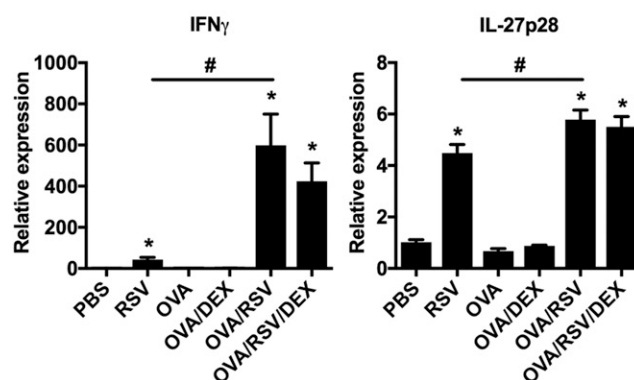


FIGURE 2. RSV infection increased production of IFN- γ and IL-27p28 in mice with AAD. (A) mRNA and protein levels of IFN- γ and IL-27p28 in the lung tissue. (B) mRNA levels of IFN- γ and IL-27p28 in isolated pulmonary macrophages. Data are presented as mean $\pm$ SEM ($n = 6$ –8 mice per group). Results are representative of three independent experiments. * $p < 0.05$ designates significant differences from PBS-treated controls, # $p < 0.05$ designates significant differences from RSV-treated group.

that IFN- γ and IL-27p28 production are significantly enhanced during RSV-induced exacerbation of AAD, with pronounced expression in pulmonary macrophages. Furthermore, the generation of these two cytokines is steroid resistant.

Blocking IFN- γ function suppresses AHR, macrophage, and neutrophil infiltration and IL-27p28 expression during RSV-induced exacerbation of AAD

We have previously identified that IFN- γ and IL-27 (by the direct delivery of these cytokines to the lung) cooperatively and critically regulate the development of macrophage-dependent steroid-resistant inflammatory pathways that underpin AHR (40, 41). To

determine whether these pathways also operated during RSV-induced exacerbation of AAD, we initially examined the role of IFN- γ by neutralizing its function with mAb treatment. IFN- γ neutralization completely suppressed RSV-induced exacerbation of AHR (which we have previously shown to be steroid resistant) (29), compared with isotype treatment control (Fig. 3A). This suppression occurred despite a slight increase in RSV levels in the lung (Fig. 3B). Neutralization of IFN- γ significantly suppressed the infiltration of macrophages, neutrophils, and lymphocytes into the airways but had no effect on the number of eosinophils (Fig. 3C). Furthermore, neutralization of IFN- γ by mAb treatment (anti-IFN- γ group) significantly increased histopathological scores, but not PAS scores, when morphological features were

compared between the OVA/RSV/anti-IFN- γ group and the OVA/RSV/isotype Ab group (Fig. 3D).

Recently, we have shown that TNF- α and MCP-1 play important roles in the development of RSV-induced exacerbation of AAD (29). Thus, we next determined whether IFN- γ neutralization impacts on the production of IFN- γ , IL-27p28, TNF- α , and MCP-1. First, we confirmed that treatment with anti-IFN- γ Ab completely neutralized OVA/RSV-induced upregulation of IFN- γ protein in lung, although it did not affect IFN- γ mRNA expression (Fig. 4A). Neutralization of IFN- γ decreased IL-27p28 mRNA levels and with a slight, but not significant, decrease in protein levels (Fig. 4B). TNF- α and MCP-1 mRNA or protein levels were not affected by anti-IFN- γ Ab treatment (Fig. 4C, 4D). Taken

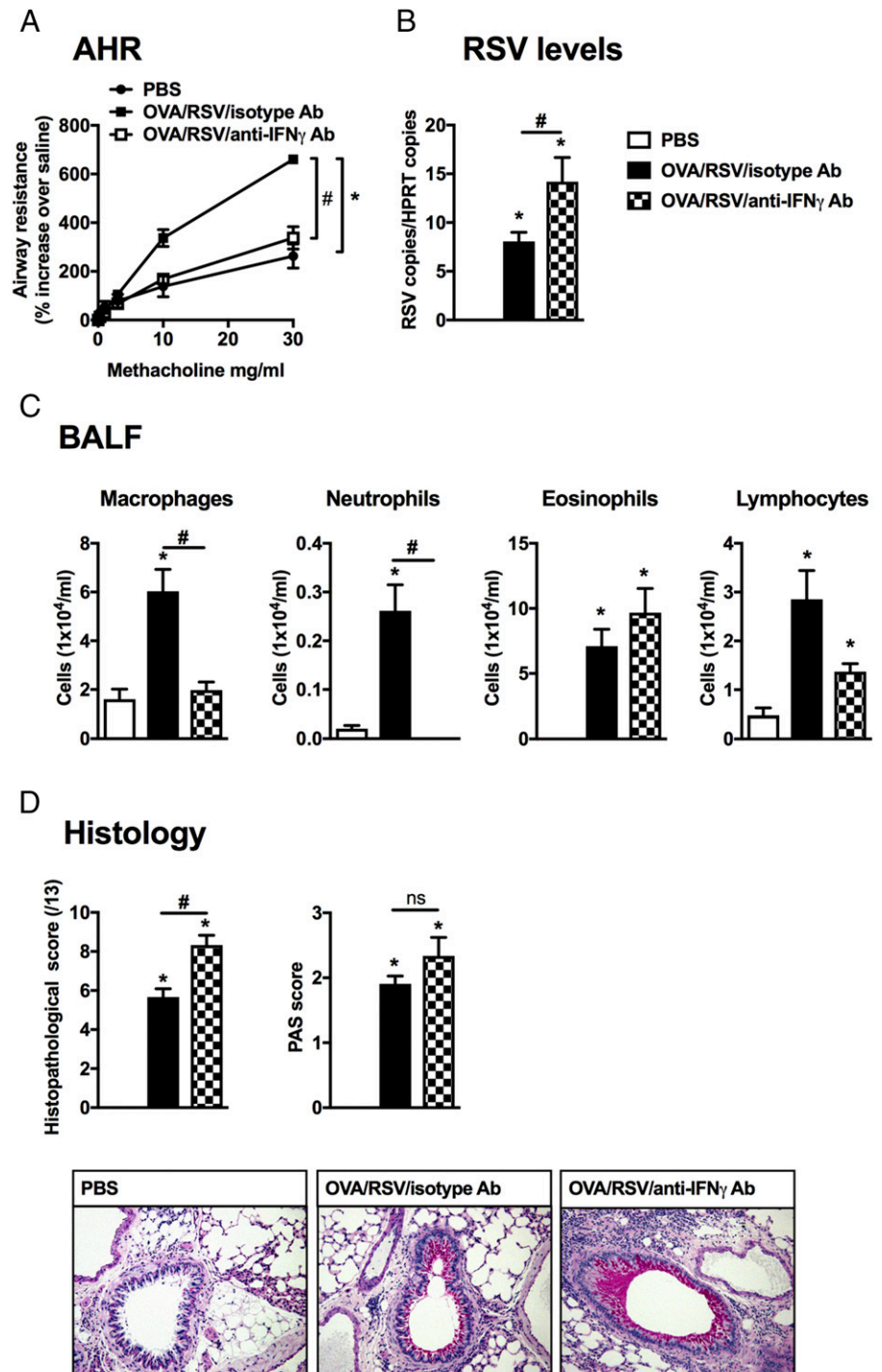
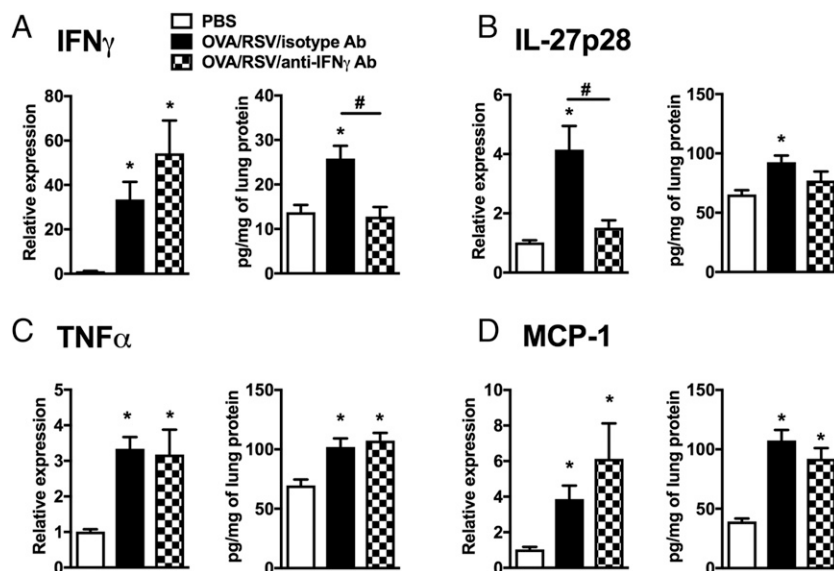


FIGURE 3. Blockade of IFN- γ function suppressed AHR and inflammatory cell infiltration in RSV-infected mice with AAD. OVA/RSV-treated mice were administered with anti-IFN- γ Ab or isotype control Ab on days 20 and 22. (A) AHR and (B) RSV levels in lung tissue were assessed on day 24. (C) Differential cell counts in BALF for macrophages, neutrophils, eosinophils, and lymphocytes were performed by light microscopy. (D) Inflammatory infiltrates and mucus-producing cells in lung. PAS stained. Original magnification $\times 10$. Data are presented as mean $\pm$ SEM ($n = 6$ –8 mice per group). * $p < 0.05$ designates significant differences from PBS-treated controls, # $p < 0.05$ designates significant differences from isotype Ab group. ns, nonsignificant.

FIGURE 4. Blockade of IFN- γ function had no impact on the production of IL-27p28, TNF- α , or MCP-1 in the lung of RSV-treated mice with AAD. OVA/RSV-treated mice were administrated with anti-IFN- γ Ab or isotype control Ab on days 20 and 22, and samples were collected on day 24. **(A)** IFN- γ , **(B)** IL-27p28, **(C)** TNF- α , and **(D)** MCP-1. Data are represented as mean $\pm$ SEM ($n = 6-8$ mice per group). * $p < 0.05$ designates significant differences from PBS-treated controls, # $p < 0.05$ designates significant differences from isotype Ab group.



together, our results suggest that IFN- γ contributes to RSV-induced AHR and macrophage/neutrophil infiltration; however, neutralization did not significantly affect the protein levels of IL-27p28, TNF- α , or MCP-1.

Blocking IL-27 function suppresses AHR in RSV-induced exacerbation

We next examined the role of IL-27 in RSV-induced exacerbation of AAD by administrating IL-27p28 Ab. Neutralizing IL-27p28 significantly suppressed AHR, compared with isotype Ab treatment (Fig. 5A), but did not affect the level of RSV in the lung (Fig. 5B). The levels of BALF cells, including macrophages, neutrophils, eosinophils, and lymphocytes, were not affected by anti-IL-27p28 Ab treatment (Fig. 5C).

In lung tissue, anti-IL-27p28 Ab treatment reduced IL-27p28 protein levels with a slight, but not significant, decrease in mRNA levels (Fig. 6A). Administration of anti-IL-27p28 Ab did not affect the mRNA or protein levels of IFN- γ , TNF- α , or MCP-1

(Fig. 6B–D). Collectively, these data indicate that IL-27p28 plays a role in the induction of AHR in RSV-induced exacerbation of AAD, but not by regulating key inflammatory mediators or the infiltration of inflammatory cells into the lung.

TNF- α and/or MCP-1 stimulate IFN- γ and IL-27p28 production by pulmonary macrophages

In our previous study, we demonstrated key roles for TNF- α , MCP-1, and macrophages in RSV-induced exacerbation of AAD (29). In this investigation, we also showed that pulmonary macrophages produced high levels of IFN- γ and IL-27p28 following RSV infection on the background of AAD (Fig. 2B). We therefore determined whether these factors regulate the expression of IFN- γ and IL-27p28 in pulmonary macrophages. Macrophages isolated from untreated lung tissue were stimulated with TNF- α and/or MCP-1 for 12 h. Stimulation with TNF- α increased the mRNA and protein levels of IFN- γ and IL-27p28 in macrophages and the culture supernatant, respectively (Fig. 7A, B). MCP-1 stimulation

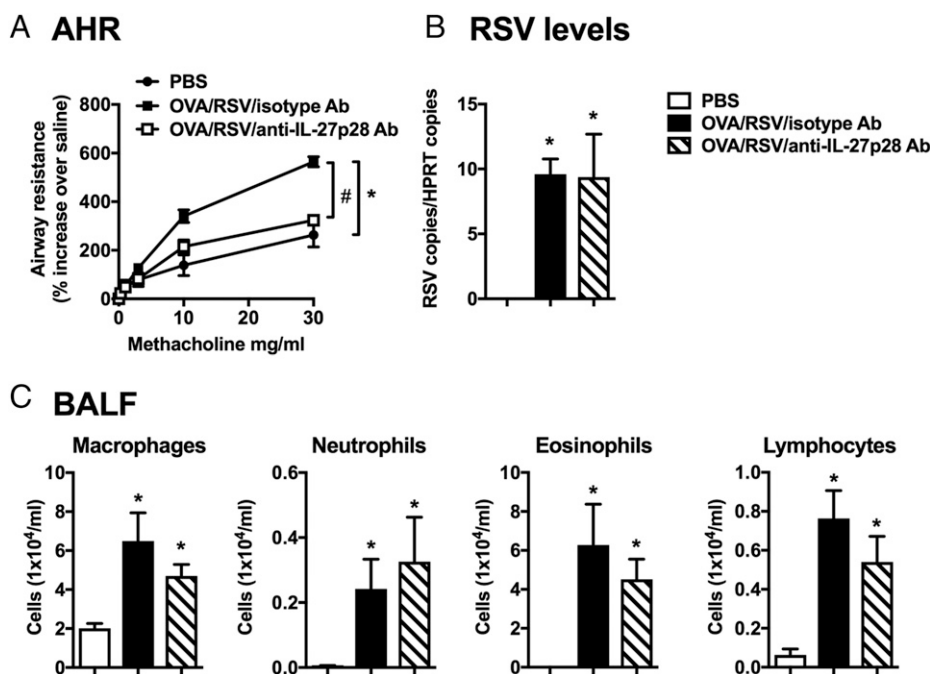
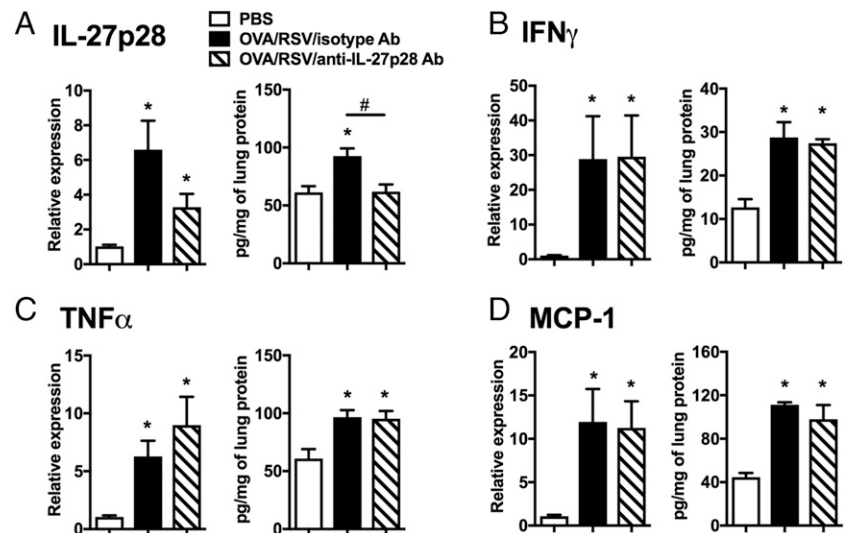


FIGURE 5. Blockade of IL-27 function suppressed AHR in RSV-infected mice with AAD. OVA/RSV-treated mice were administrated with anti-IL-27p28 Ab or isotype control Ab on days 20 and 22. **(A)** AHR and **(B)** RSV levels in lung tissue were assessed on day 24. Differential cell counts in **(C)** BALF for macrophages, neutrophils, eosinophils, and lymphocytes were performed by light microscopy. Data are represented as mean $\pm$ SEM ($n = 6-8$ mice per group). * $p < 0.05$ designates significant differences from PBS-treated controls, # $p < 0.05$ designates significant differences from isotype Ab group.

FIGURE 6. Blockade of IL-27 function had no impact on the production of IFN- γ , TNF- α , or MCP-1 in the lung of RSV-infected mice with AAD. OVA/RSV-treated mice were administrated with anti-IL-27p28 Ab or isotype control Ab on days 20 and 22, and samples were collected on day 24. **(A)** IL-27p28, **(B)** IFN- γ , **(C)** TNF- α , and **(D)** MCP-1. Data are represented as mean $\pm$ SEM ($n = 6-8$ mice per group). * $p < 0.05$ designates significant differences from PBS-treated controls, # $p < 0.05$ designates significant differences from the isotype Ab group.



was also sufficient to increase the mRNA and protein levels of IFN- γ and IL-27p28 (Fig. 7A, 7B). Similarly, a combined stimulation with TNF- α and MCP-1 also increased IFN- γ and IL-27p28 mRNA and protein levels in culture supernatant, but the effect was not additive (Fig. 7A, 7B). This suggests that TNF- α and MCP-1 operate similar pathways to enhance production of these factors.

Next, we determined whether the expression of IFN- γ and IL-27p28 was affected by neutralizing TNF- α or MCP-1, and by the depletion of pulmonary macrophages. Treatment with anti-TNF- α Ab or depletion of macrophages by 2-CA administration suppressed the expression of both IFN- γ and IL-27p28 mRNA levels (Fig. 8A, 8C). By contrast, treatment with anti-MCP-1 Ab inhibited only IL-27p28, but not IFN- γ , mRNA expression

(Fig. 8B). Taken together, our data demonstrate that TNF- α and/or MCP-1 drive pulmonary macrophages to produce IFN- γ and IL-27p28, underpinning the mechanism of RSV-induced exacerbation of steroid-resistant AHR and airway inflammation in AAD.

Discussion

Numerous clinical studies have highlighted a unique role of aberrant innate immune responses in the pathogenesis of virus-induced exacerbation of asthma (36, 37, 59, 60). Previously, we have shown that TNF- α /MCP-1-regulated pulmonary macrophages activation is important in the development of RSV-induced steroid-resistant exacerbation of AHR and airway inflammation (29). In another study, we have also identified that IFN- γ and IL-27 cooperate to induce steroid-resistant inflammatory pathways

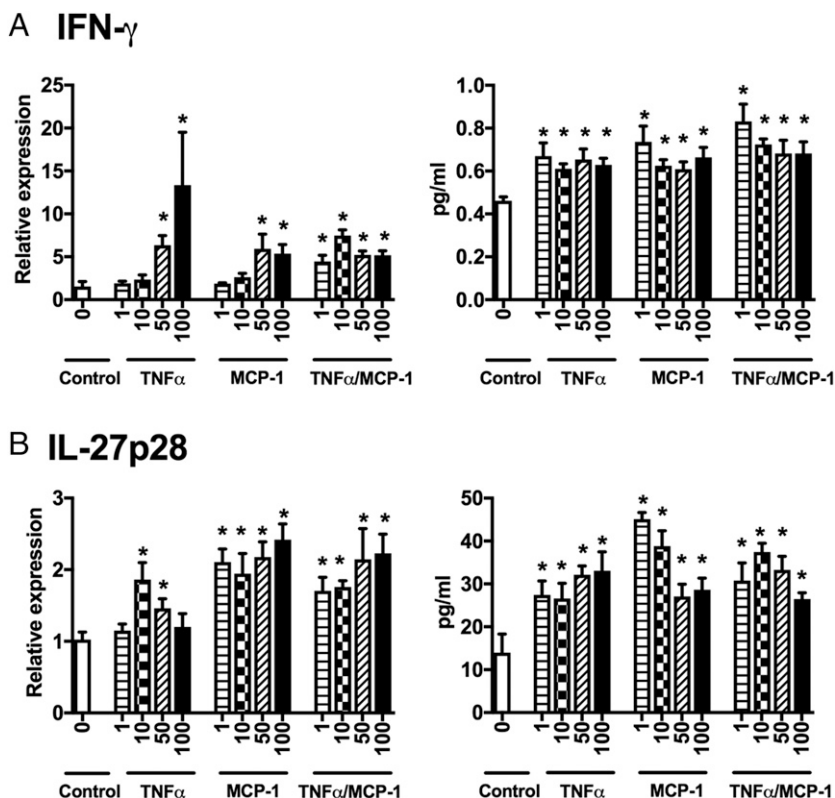


FIGURE 7. TNF- α - and/or MCP-1-stimulated pulmonary macrophages produce increased levels of IFN- γ and IL-27p28. Pulmonary macrophages were isolated from naive lung tissues and then stimulated with different concentrations (0, 1, 10, 50, or 100 ng/ml) of TNF- α , MCP-1, or a combination of both cytokines. The mRNA and protein levels of **(A)** IFN- γ and **(B)** IL-27p28 were quantitated 12 h after treatment. Data are represented as mean $\pm$ SEM ($n = 5$ per group). * $p < 0.05$ versus unstimulated control.

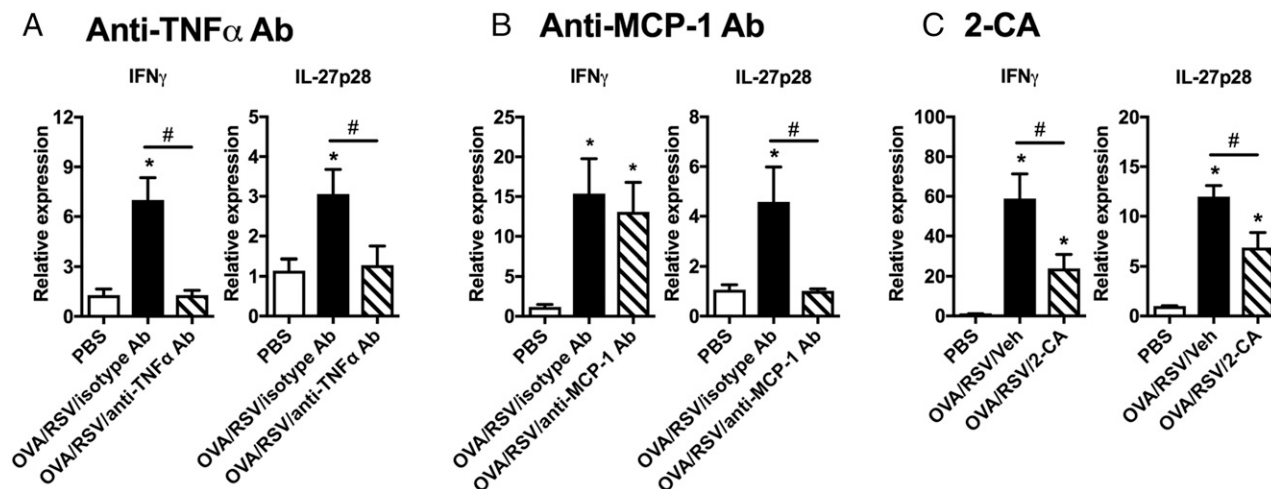


FIGURE 8. Neutralization of TNF- α or MCP-1 or depletion of pulmonary macrophages inhibited the expression of IFN- γ and IL-27p28. OVA/RSV-treated mice were administrated with anti-TNF- α or anti-MCP-1 Abs or isotype control Ab on days 20 and 22. In some experiments, pulmonary macrophages in OVA/RSV-treated mice were depleted by 2-CA treatment on days 20 and 22. Lung samples were collected on day 24. The levels of IFN- γ and IL-27p28 mRNAs following treatments with (A) anti-TNF- α Ab, (B) anti-MCP-1 Ab, or (C) 2-CA. Data are represented as mean $\pm$ SEM ($n = 6-8$ mice per group). * $p < 0.05$ designates significant differences from PBS-treated controls, # $p < 0.05$ designates significant differences from isotype Ab or vehicle groups.

(40). In this model, delivery of IFN- γ and IL-27 (but not alone) induced exaggerated steroid-resistant AHR and inflammation that was dependent on macrophage activation (40). Therefore, we extend our investigations to define whether IFN- γ and IL-27 play roles in the development of RSV-induced exacerbation of AAD and how IFN- γ , IL-27, TNF- α , and MCP-1 interact to trigger the exacerbation.

In this study, we observed significant increases in IFN- γ and IL-27 levels in lung tissues and in isolated pulmonary macrophages following RSV inoculation of mice with AAD. The expression of IFN- γ and IL-27 was resistant to Dex treatment, which potentially links both cytokines to the development of asthma exacerbations. Indeed, high levels of serum IFN- γ have been strongly associated with the decline of lung function in asthmatic patients (61), and elevated levels of IL-27 have been found in BALF cells of severe asthmatic patients, who are insensitive to steroid treatment (39). Furthermore, the levels of both cytokines are significantly higher in the sputum of patients with neutrophilic but not eosinophilic asthma; the former group of asthma patients often poorly responds to glucocorticoids (40). Our observation is also corroborated by those clinical studies showing that increased IFN- γ expression in sputum cells is correlated with virus-associated asthma exacerbations (36). In addition, our results support other studies that suggest IFN- γ critically participate in the development of asthma, especially in the steroid-resistant forms of the disease (40, 41, 45, 57). Collectively, clinical and experimental data suggest that IFN- γ by cooperating with IL-27 may play an important role in the induction of steroid resistance in asthma following viral infection-induced exacerbations.

We treated mice with anti-IFN- γ Ab to further define the role of this cytokine in RSV-induced exacerbation of AAD. Although we have previously demonstrated that IFN- γ and IL-27 act synergistically to drive steroid-resistant AHR and airway inflammation (by direct delivery of these factors to the lung) and are linked to neutrophilic asthma, we have not investigated their roles in the pathogenesis of virus-induced asthma exacerbations. In our model of RSV-induced exacerbation of AAD, we observed that anti-IFN- γ Ab treatment completely abolished steroid-resistant exaggerated AHR and reduced the levels of infiltrating macrophages and neutrophils to almost baseline levels (PBS group). Furthermore, Kumar and colleagues (62) have also reported a similar observation that

neutralizing IFN- γ significantly suppressed AHR in a mouse model of chronic asthma. Interestingly, neutralization of IFN- γ increased levels of RSV in the lung tissue. This observation is consistent with studies showing an important role of IFN- γ in viral clearance (63). Furthermore, anti-IFN- γ mAb treatment led to higher levels of inflammatory infiltrates in lung and less numbers of macrophages and neutrophils in BALF, suggesting that this cytokine promotes the migration of inflammatory cells from lung tissue to BALF. Collectively, our data suggest that IFN- γ plays an important role in the development of steroid-resistant exaggerated AHR and airway inflammation in response to RSV infection.

Although the role of IL-27 in the pathogenesis of asthma exacerbations is largely unknown, there is evidence that IL-27 promotes LPS-induced proinflammatory cytokines (IL-6, TNF- α , and MIP-1) in human monocytes (48), and its expression is associated with the severity of asthma (39). Increased levels of IFN- γ and LPS are linked to the severity of asthma and in some patients with steroid resistance (38, 61, 64, 65). We have also previously shown that IFN- γ /LPS-stimulated macrophages produce significantly higher levels of IL-27, and that the subsequent interplay between IFN- γ and IL-27 (by autocrine and paracrine mechanisms) underpins the development of steroid-resistant AHR and airway inflammation (40). In this study, we found that blocking IL-27 significantly inhibited the onset of RSV-induced exaggerated AHR, although there was no effect on the infiltration of inflammatory cells or the production of proinflammatory mediators (e.g., IFN- γ , TNF- α , and MCP-1). This observation is in agreement with our previous work that IL-27 neutralization completely suppresses steroid-resistant AHR, but not airway inflammation, induced by IFN- γ /LPS treatment (40). In addition, RSV infection had no impact on the infiltration of CD19⁺ B cells, type 2 innate lymphoid cells, and CD4⁺ and CD8⁺ T cells in the lung on day 24 (data not shown). This suggests the predominant role for IL-27 is downstream of macrophage activation for the induction of AHR.

Accumulating clinical and experimental evidence highlights a potentially important role for pulmonary macrophages in the pathogenesis of asthma and asthma exacerbations, especially in the steroid-resistant form of the disease (31, 40, 64, 66–68). Pulmonary macrophages play a central role in host defense against respiratory infections. The persistent activation of these cells in the

setting of chronic allergic inflammation may lead to their dysfunction (limitations in the ability to clear pathogens) and a proinflammatory environment that drives exaggerated AHR and airway inflammation (63, 69). Recently, we have identified that TNF- α /MCP-1-activated pulmonary macrophages orchestrate the development of RSV-induced exacerbation of AAD in a mouse model of AAD (29). In this study, we extend these observations to demonstrate that TNF- α and/or MCP-1 stimulation of pulmonary macrophages upregulates the production of IFN- γ and IL-27. Furthermore, anti-TNF- α Ab treatment or depletion of macrophages in our model of RSV-induced exacerbation downregulated the expression of not only IFN- γ , but also IL-27. Anti-MCP-1 Ab treatment decreased the production of IL-27, but not IFN- γ , in vivo. Based on these findings, we speculate that RSV infection initially induces the production of TNF- α and MCP-1 to stimulate pulmonary macrophage recruitment and activation in the lung, which in turn leads to an increase in the production of IFN- γ and IL-27 by these cells (and potentially others). Thus, our data suggest that IFN- γ and IL-27 play critical roles in the development of RSV-induced exacerbation of AAD and downstream of TNF- α and MCP-1 production. Blocking the function of any of these factors reduces the severity of exacerbations by suppressing steroid-resistant AHR and/or airway inflammation. Notably, we and other have shown pulmonary macrophages in vitro are capable of producing IL-27, in response to inflammatory stimulants (40, 43). Furthermore, it has been long recognized that macrophages secrete IFN- γ upon cytokine stimulation (70, 71). Indeed, the activation status of macrophages is a product of the complex microenvironment (72). These cells exhibit extraordinary plasticity and can transform their physiology in response to environmental signals (73). Although the versatile function of macrophages makes the task of defining their distinct subpopulations difficult, we believe that further investigation with macrophage plasticity after allergic/infectious Ag challenge are needed to better understand the relationships between these cells and asthma pathogenesis.

Although there is clinical and experimental evidence linking IFN- γ , IL-27, TNF- α , or MCP-1 to the pathogenesis of asthma exacerbations and steroid-resistant inflammatory mechanisms, no double-blinded placebo-controlled study has been performed by targeting these molecules. Of these four factors, targeting TNF- α has shown limited efficacy in some preliminary studies (74, 75). For example, treatment with soluble TNF- α receptor fusion protein (etanercept) was slightly effective in controlling systemic inflammation as measured by serum albumin and C-reactive protein; however, it failed to improve lung function and exacerbation rates in patients with severe refractory asthma (74). Furthermore, in an unrandomized, unblinded case series of seven patients with severe steroid-resistant asthma, anti-TNF- α Ab treatment also yielded promising outcomes by improving asthma management and reducing exacerbations (75). Further examination of the mechanisms of interplay between these factors (e.g., IFN- γ , IL-27, TNF- α , and MCP-1) may provide insight into new approaches for the treatment of virus-induced asthma exacerbations.

In summary, we have described key roles for IFN- γ and IL-27 in the pathogenesis of RSV-induced steroid-resistant exacerbation of AAD. Our findings suggest that targeting signaling pathways activated by the cooperative interaction between IFN- γ /IL-27 could be a potential approach for the treatment of RSV-induced exacerbation of asthma. Furthermore, our data indicate that aberrant innate immune responses (proinflammatory mediators and macrophages) are major contributors to RSV-induced steroid-resistant exacerbation of AAD.

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Disclosures

The authors have no financial conflicts of interest.

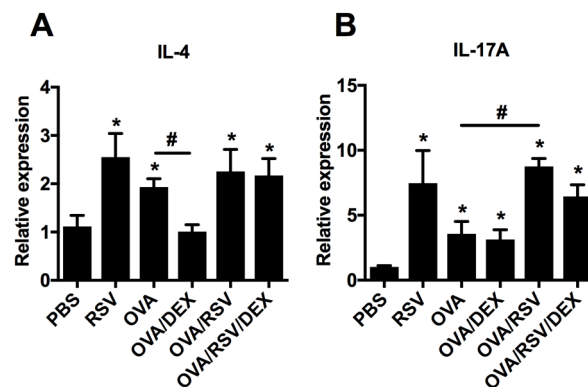
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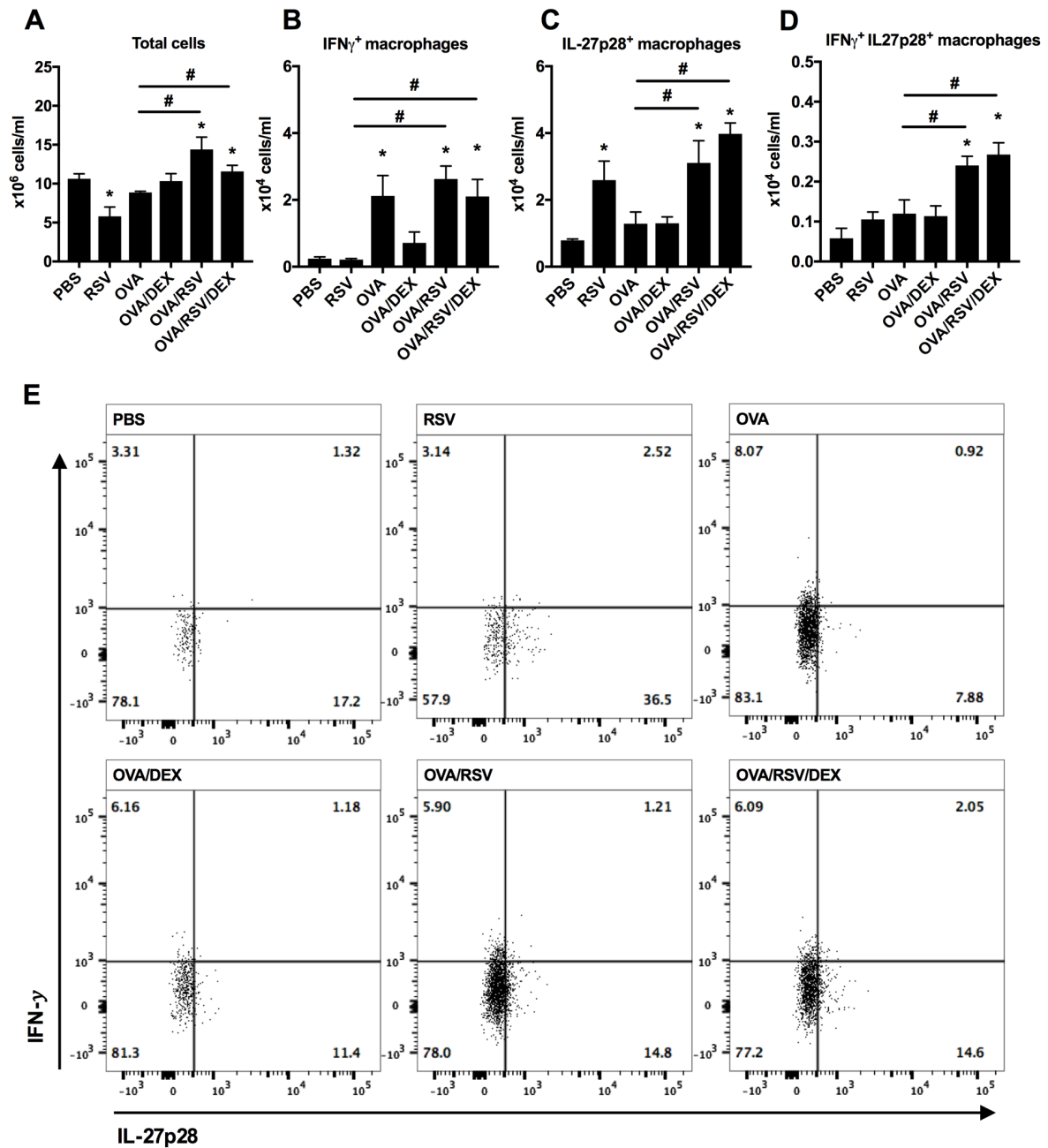
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Supplementary Data



Supplementary Figure 1. The effect of Dex treatment on the expression of IL-4 and IL-17A in the lung of mice with AAD following RSV infection. BALB/c mice were sensitised i.p with OVA or PBS on day 0 and exposed to OVA aerosol on days 13-16. Some mice were treated with Dex on day 18 and 20, and inoculated with RSV on day 19. Levels of (A) IL-4 and (B) IL-17A mRNA in lung were assessed by qPCR. Data are presented as mean $\pm$ SEM (n=6-8 mice/group). Results are representative of three independent experiments. *P<0.05, designates significant differences from PBS-treated controls. #P< 0.05, designates significant differences between respective groups.



Supplementary Figure 2. Production of IFN- γ and IL-27p28 by lung macrophages.

Single cell suspensions from lungs were prepared and stained with surface marker and then with anti-IFN- γ and anti-IL-27p28 antibodies. (A) Total cells in the lung tissues (B) IFN- γ ⁺ macrophages, (C) IL-27p28⁺ macrophages and (D) IFN- γ ⁺IL-27p28⁺ macrophages were gated as CD11b⁺F4/80⁺FS^{high}SS^{high}IFN- γ ⁺, F4/80⁺CD11b⁺FS^{high}SS^{high}IL-27p28⁺ and CD11b⁺F4/80⁺FS^{high}SS^{high}IFN- γ ⁺IL-27p28⁺, respectively. (E) Representative dot plots of

intracellular cytokine staining in lung F4/80⁺CD11b⁺ cells from PBS, RSV, OVA, OVA/Dex, OVA/RSV and OVA/RSV/Dex treated mice on day 24. Data are represented as mean $\pm$ SEM (n=6 mice/group). *P<0.05, designates significant differences from PBS-treated controls. #P<0.05, designates significant differences between respective groups.