

Elevated Macrophage Inflammatory Protein 1 α and Interleukin-17 Production in an Experimental Asthma Model Infected with Respiratory Syncytial Virus

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Key Words

Eosinophils · Respiratory syncytial virus · Cytokines · Mice

Abstract

Background: Respiratory syncytial virus (RSV) infection is associated with both the development and exacerbation of bronchial asthma. We examined eosinophil infiltration and the cytokine profiles of both airway and peripheral blood in antigen-sensitized mice infected with RSV to investigate the pathogenesis of exacerbations of asthma due to RSV infection. **Methods:** Ovalbumin (OVA)-sensitized mice were challenged by OVA inhalation 3 times and then infected with RSV [10^5 TCID₅₀ (50% of tissue culture infectious dose)/25 g body weight] or mock infection immediately after the last challenge. Animals from each group, namely, the control (PBS instead of OVA inhalation plus mock infection), RSV (PBS plus RSV), OVA (OVA plus mock) and OVA/RSV (OVA plus RSV) were analyzed. Analysis included evaluation of airway responsiveness to methacholine, pathological findings in the airway by hematoxylin and eosin (HE) and Luna staining, bronchoalveolar fluid (BALF) and peripheral leukocytes counts, and concentrations of multiple cytokines/chemokines in both BALF and serum. **Results:** Airway responsiveness was significantly enhanced in the OVA and OVA/RSV

groups compared with the control group. Levels of tissue and BALF eosinophils were higher in the OVA and OVA/RSV groups than in the RSV or control group. Significantly higher levels of macrophage inflammatory protein (MIP)-1 α in BALF were observed in the OVA/RSV group compared with the 3 other groups. Production of serum IL-17 was also significantly elevated in the OVA/RSV group compared with the control or OVA group. **Conclusions:** These findings suggest that MIP-1 α and IL-17 may play important roles in acute exacerbation of asthma induced by RSV in an animal model.

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Introduction

Viral infection induces both the development and exacerbation of bronchial asthma [1]. Respiratory syncytial virus (RSV) is a leading cause of serious lower respiratory tract infection, including acute bronchiolitis in infants. RSV infection also exacerbates recurrent wheezing attacks in patients with established asthma [2].

Eosinophils are proinflammatory granulocytes implicated in host immunity to parasitic infections and allergic diseases such as asthma, allergic rhinitis and atopic dermatitis [reviewed in 3]. Appropriate stimuli such as

immunoglobulins [4], cytokines [5] and lipid mediators, including leukotrienes [6] and platelet-activating factor [7], induce degranulation of toxic eosinophil granule proteins. Subsequent tissue damage induced by eosinophil granule proteins and superoxides contributes to the airway inflammation associated with such diseases [3, 8].

In a murine model, infection of neonatal mice with the paramyxoviruses RSV and pneumonia virus of mice interacted with ovalbumin (OVA) sensitization and challenge to increase the risk of subsequent development of an asthma-like phenotype [9]. Likewise, other studies have examined the effects of RSV infection during OVA challenge on asthma induction in mice, most of which were used by BALB/c [reviewed in 10]. RSV infection can aggravate the consequences of subsequent airway sensitization to allergen, resulting in airway hyperresponsiveness and an influx of eosinophils into the lungs [11]. However, the acute effects of postsensitization RSV infection in allergic mice used by another phenotype, C57BL/6 mice are less well investigated.

Thus, we examined the eosinophil infiltration and cytokine profiles of both airway and peripheral blood using an experimental asthma model infected with RSV in efforts to elucidate the pathogenesis of asthma exacerbation due to acute RSV infection.

Materials and Methods

Animals

Specific pathogen-free and virus-antibody-free, 5-week-old, male C57BL/6 background mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animals were bred and housed in specific-pathogen-free conditions and maintained on an OVA-free diet. The mice were used in the experiments at 5–10 weeks of age. Control mice were age- and sex-matched with the mice in each experimental group. The experimental protocol was reviewed and approved by the Laboratory Animal Care and Use Committee of Gunma Prefectural Institute of Public Health and Environmental Sciences.

Virus

RSV (A-2 strain) was obtained from the American Type Culture Collection (ATCC) VR-1540, propagated in monolayers of HEp-2 cells (ATCC, CCL-23TM) and grown in DMEM supplemented with 2% FCS. At maximum cytopathic effect, the suspension was centrifuged at 1,400 g for 30 min at 4°C to remove debris. The supernatants were layered over 50% sucrose in phosphate-buffered saline (PBS, Sigma, St. Louis, Mo., USA), and centrifuged at 65,000 g for 2 h at 4°C. Aliquots of purified RSV were stored at –80°C until required. The viral titer of the pooled purified RSV was 2.0×10^8 TCID₅₀ (50% of tissue culture infectious dose)/0.1 ml. Full inactivation of RSV was achieved by exposing a sterile glass vial containing viral suspension to an ultra violet (UV) light source (302 nm) for 16 h at 4°C as previously reported [12, 13].

Sensitization, Airway Challenge and Infection of Mice

Mice were sensitized by intraperitoneal (i.p.) injection of OVA (2.0 mg/kg) in alum or PBS alone on days 0 and 14 as previously reported with minor modifications [14]. Mice were then challenged via the airway with OVA (1% in PBS) or PBS for 30 min on days 24, 25 and 26. On day 26, mice were also infected under light anesthesia (tribromoethanol 2.5%, 0.015 ml/g body weight) by intranasal inoculation of RSV (10^5 TCID₅₀/25 g body weight). Controls were mock-infected (UV-RSV or sham inoculum) in the same way. On day 29, airway function was measured and specimens were collected for further analysis (fig. 1).

Determination of Airway Responsiveness

Airway responsiveness was assessed by an intravenous (i.v.) methacholine challenge, as previously described [15]. Mice were anesthetized by i.p. injections of sodium pentobarbital (39 mg/kg). Tracheostomy was performed and a cannula was inserted into the Y-adaptor of a small animal ventilator (MiniVent 845, Harvard Apparatus, Holliston, Mass., USA). Mice were mechanically ventilated at a tidal volume of 10 ml/kg and a respiratory rate of 150 breaths/min. Doubling incremental doses of methacholine chloride ranging from 31.3–2,000 µg/kg were given by i.v. injection and resistance was measured.

Analysis of Bronchoalveolar Lavage Fluid and Peripheral Blood

After assessment of airway responsiveness, lungs were lavaged via a tracheal tube with PBS containing 0.5% BSA (2 × 1 ml). Cells in the collected bronchoalveolar lavage fluid (BALF) were counted using a cell counter (Coulter, Hialeah, Fla., USA). Cytospin slides were stained with Diff-Quik and differentiated in a blinded fashion by counting at least 200 cells under a light microscope. Peripheral leukocytes were also stained with hematoxylin and eosin (HE) and evaluated under a light microscope.

Measurement of Serum Immunoglobulins

Serum levels of OVA-specific IgE were measured by ELISA (Shibayagi Co., Ltd., Shibukawa, Japan).

Measurement of Cytokines/Chemokines in BALF and Serum

BALF and serum cytokines/chemokines including interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13 and IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, keratinocyte chemokines (KC), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T-cell expressed and secreted (RANTES) and tumor necrosis factor (TNF)-α were determined by a multi-cytokine detection system (Bio-Rad, Hercules, Calif., USA), following the manufacturer's instructions, measured using a Luminex System (Austin, Tex., USA), and then quantified using Bio-Plex software (Bio-Rad) [12, 16].

Histological Studies

After obtaining BALF, lungs were inflated through the tracheal tube with 2 ml air, fixed in 10% formalin, and blocks of lung tissue were then prepared. Tissue sections, 5 µm thick, were affixed to microscope slides and deparaffinized. The slides were stained with HE and Luna for identification of eosinophils and examined under

a light microscope. Peripheral leukocytes were also stained with HE and evaluated under a light microscope.

Statistical Analysis

All analysis was performed by ANOVA with Tukey-Kramer honest significant difference using a statistical software package (SPSS for Windows, version 18.0, SPSS Japan Inc., Tokyo, Japan). Statistically significance was set at $p < 0.05$ (two-sided).

Results

Airway Responsiveness in Allergen-Sensitized and Challenged Mice Infected with RSV

Mice that had been sensitized and challenged with OVA (OVA group) developed significant changes in lung resistance (RL) (fig. 2) when compared with control mice. Mice with both OVA sensitization/inhalation and RSV infection (OVA/RSV group) also developed significant increases in RL when compared with the controls (fig. 2). Although mice with RSV infection (RSV group) displayed an increase in RL, the results were not significantly different from the controls (fig. 2).

BALF and Peripheral Blood Analysis of Cells

The percent of total cells for eosinophils in BALF in the OVA and OVA/RSV groups was significantly increased compared with the control or RSV group (fig. 3a), but no eosinophils were detected in the BALF of the control or RSV groups. In peripheral blood, the significant difference was shown in the percent of total cells for eosinophils in the OVA group when compared with RSV group (fig. 3b).

Histopathology

No eosinophils were detected in the control groups (fig. 4a, c). A few eosinophils were detected by HE and Luna staining (fig. 4b, d) in the RSV group. In contrast, there were prominent numbers of eosinophils in the OVA and OVA/RSV groups (fig. 4e, f). Luna staining of the sections clearly showed an increase in eosinophils in these groups (fig. 4g, h).

Immunoglobulins

Total IgE concentrations in serum were significantly higher in the OVA group than in the control or RSV-infected groups (data not shown). Although there was a tendency for the OVA/RSV group to show increased antibody levels, there were no significant differences between the groups (data not shown).

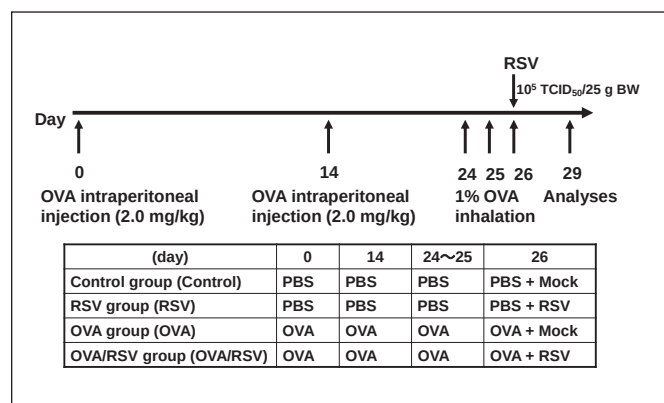


Fig. 1. Timeline of experimental protocol. Mice were sensitized by intraperitoneal injection of OVA or PBS on days 0 and 14, and exposed to OVA or PBS inhalation treatment from day 24 to day 26. On day 26, mice were infected with RSV or mock infection. They were killed on day 29 to perform further analysis. The groups shown are as follows: control (PBS sensitization/inhalation instead of OVA and mock infection instead of RSV), RSV (PBS sensitization/inhalation instead of OVA and RSV infection), OVA (OVA sensitization/inhalation and mock infection instead of RSV) and OVA/RSV (OVA sensitization/inhalation and RSV infection). BW = Body weight.

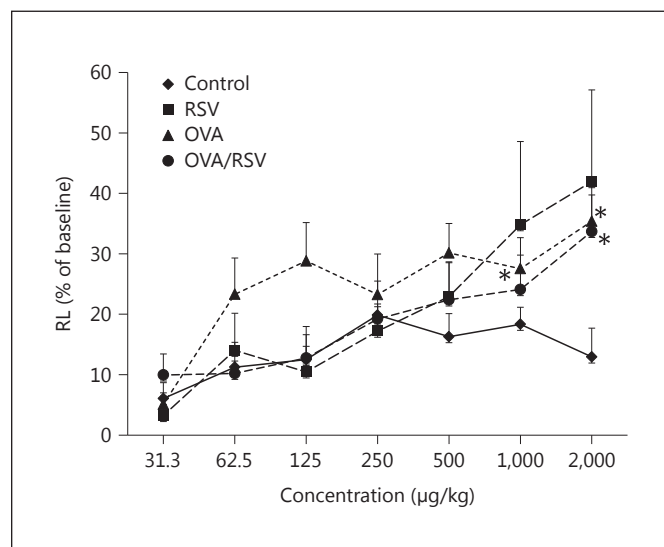
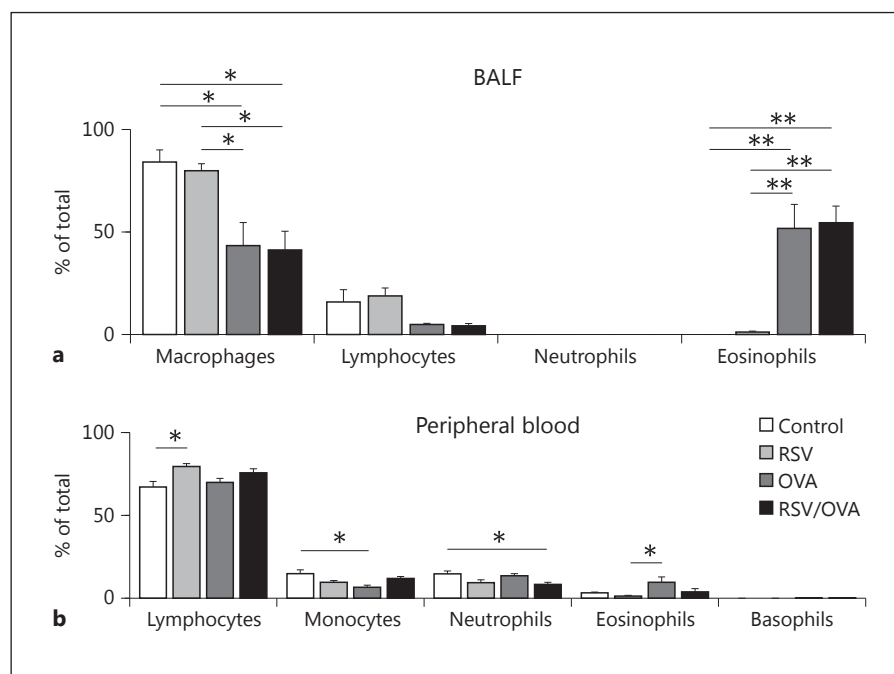


Fig. 2. Airway responsiveness to methacholine in OVA-sensitized and/or RSV-infected mice. Mice were sensitized and challenged with OVA or PBS and either infected with RSV or mock-infected. Airway responsiveness was monitored by measuring RL as described in Materials and Methods. The results for each group are expressed as means $\pm$ SEM ($n = 4$ per group). Significant differences compared with control group: * $p < 0.05$.

Fig. 3. Cellular composition of BALF (a) and peripheral blood (b). The results for each group shown in figure 1 are expressed as % of total (means $\pm$ SEM, n = 4 per group). Total cells of BALF and peripheral blood were as follows: (BALF) control group, 5.93 ± 1.36 ; RSV group, 5.46 ± 0.95 ; OVA group, 18.9 ± 1.85 ; OVA/RSV group, 13.5 ± 1.46 ; (peripheral blood) control group, 26.6 ± 4.33 ; RSV group, 16.6 ± 4.43 ; OVA group, 14.7 ± 2.37 ; OVA/RSV group, 11.6 ± 3.22 ($\times 10^5/\text{ml}$, means $\pm$ SEM, n = 4 per group). Significant differences between the groups: * p < 0.05; ** p < 0.01.



Cytokines/Chemokines of BALF and Serum

IL-4 and KC production in BALF was significantly elevated in the OVA group compared to the control or RSV groups (fig. 5a, g). A similar tendency was observed for IL-5 and IL-13 in BALF but did not reach significance (fig. 5b, c). In contrast, IFN- γ levels were elevated only in the RSV group, but again this was not significant (fig. 5d). Notably, significantly higher levels of MIP-1 α in BALF were observed in the OVA/RSV group compared to the other 3 groups (fig. 5f). IL-12(p40) in BALF was also elevated in the OVA and OVA/RSV groups compared with the control or RSV group (fig. 5e). However, there were no differences between the OVA and OVA/RSV groups (fig. 5e).

Serum IL-5 was significantly increased in the OVA groups compared with the control or RSV group (fig. 5h), and IL-17 was significantly elevated in the OVA/RSV group compared with the control or OVA group (fig. 5i). Interestingly, serum IL-10 was significantly increased in the RSV group compared with the other 3 groups (fig. 5j).

The levels of other cytokines/chemokines in both BALF and serum including IL-1 α , IL-1 β , IL-2, IL-3, IL-6, IL-9, IL-12(p70) and IL-13, eotaxin, G-CSF, GM-CSF, MCP-1, MIP-1 β , RANTES and TNF- α were not significantly different between the groups.

Discussion

In this study, we found that airway responsiveness and eosinophils of airway tissue and BALF were higher in OVA-sensitized/challenged C57BL/6 mice than in control mice. We also found that the production of IL-4 in BALF and IL-5 in serum were elevated compared with controls. However, IFN- γ was not elevated in either sample. The results were largely consistent with previous reports [17–20]. These observations suggest important roles for a number of Th2 cytokines, including IL-4, IL-5 and IL-13, in the development of allergic asthma in humans and increased airway responsiveness in experimental models.

Next, we examined the effects of single RSV infection in OVA-sensitized/challenged C57BL/6 mice. We found enhanced airway responsiveness and marked airway tissue and BALF eosinophilia, as has been previously reported in BALB/c mice [11, 21]. A key finding of this study was that MIP-1 α and IL-17 are involved in the RSV-infected asthma model. Chemokines such as MIP-1 α , RANTES and eotaxin play important roles in a variety of immune and inflammatory responses, and specifically eosinophil infiltration [22]. Similar results were reported by Matsuse et al. [23] and showed that primary (single) and recurrent RSV infection in *Dermatophagoides farinae* (Df)-sensitized BALB/c mice increased eosinophil infiltration and the expression of MIP-1 α , RANTES and eo-

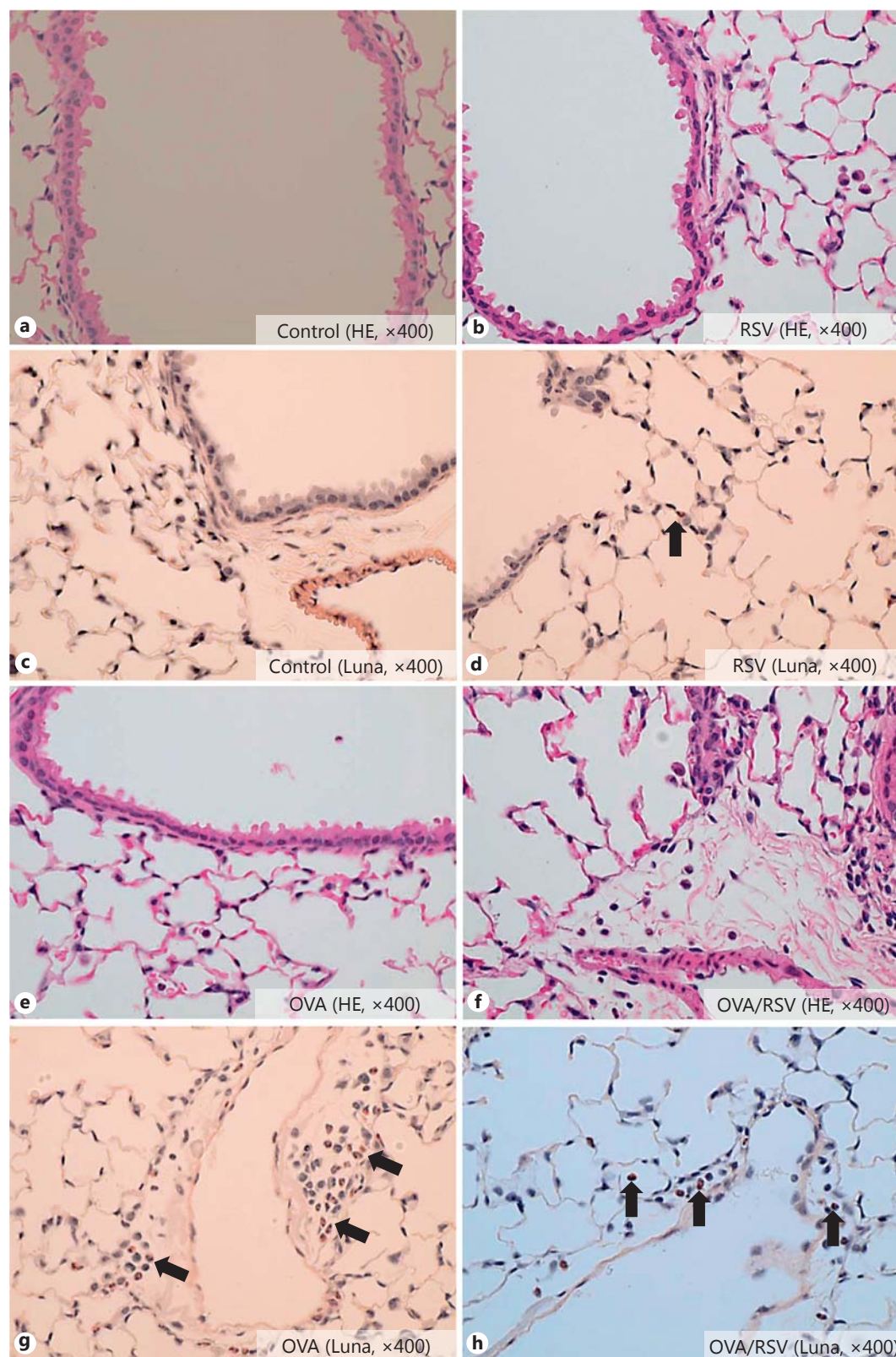


Fig. 4. Histological sections of murine lungs stained by HE and Luna. Representative sections from each group are shown. Arrows show eosinophils.

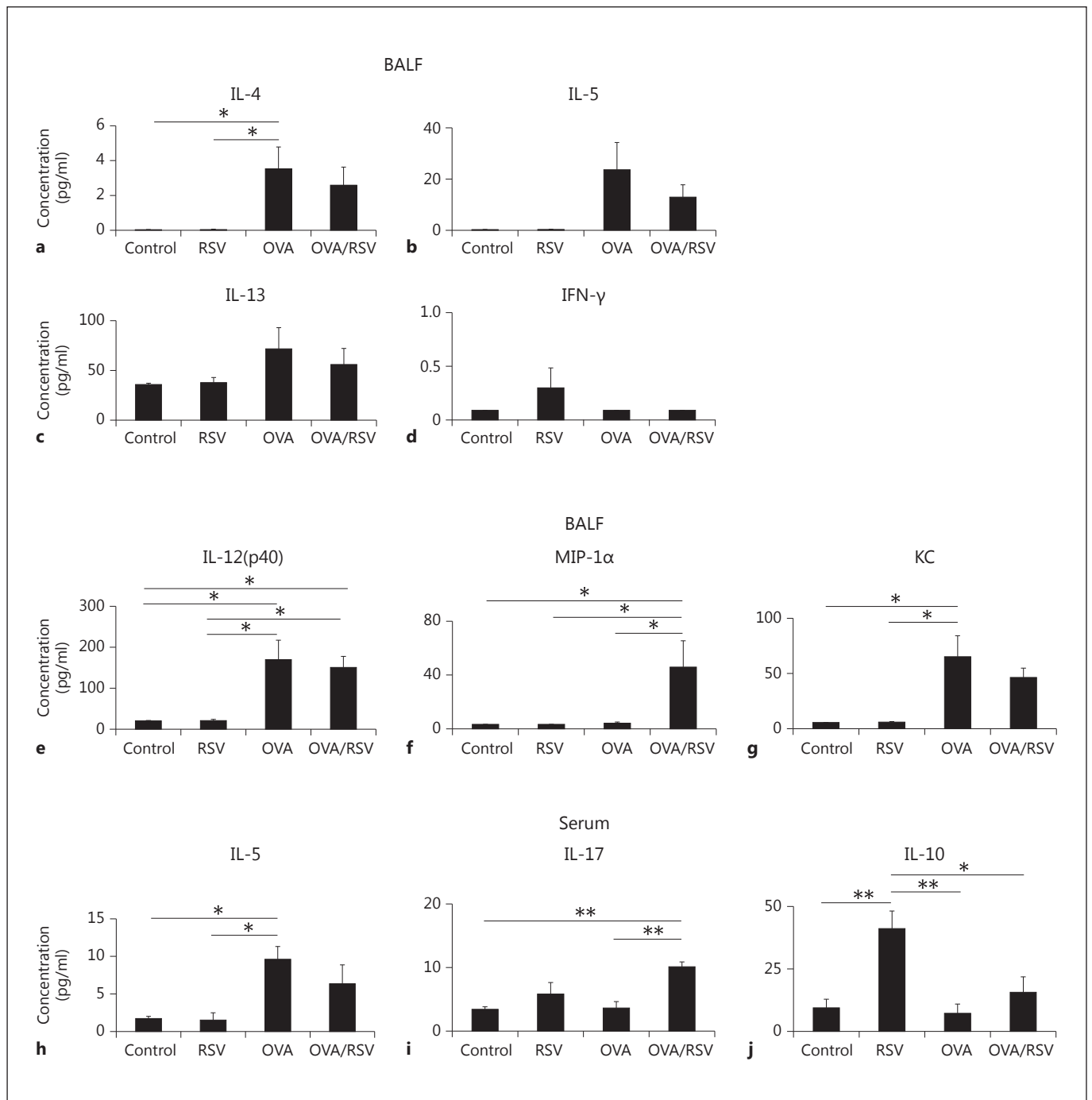


Fig. 5. The production levels of cytokines/chemokines in each group are shown as follows: **a** IL-4 in BALF. **b** IL-5 in BALF. **c** IL-13 in BALF. **d** IFN- γ in BALF. **e** IL-12(p40) in BALF. **f** MIP-1 α in BALF. **g** KC in BALF. **h** IL-5 in serum. **i** IL-17 in serum. **j** IL-10 in serum. The results for each group are expressed as means $\pm$ SEM (n = 4 per group). Significant differences between the groups: * p < 0.05; ** p < 0.01.

taxin in lung tissue. They also found that the production of both MIP-1 α and RANTES increased in single RSV infection in non-*Df*-sensitized mice and in *Df*-sensitized mice without RSV infection. Moreover, MIP-1 α production was augmented in recurrent RSV infection in *Df*-sensitized and nonsensitized mice. Another report by Jafri et al. [24] demonstrated that single RSV infection induced multiple cytokine/chemokine production of IL-4, IL-6, IL-10, TNF- α , IFN- γ , KC, MIP-1 α , RANTES, eotaxin and MIG (CXCL9), which peaked between days 0 and 5 after RSV inoculation in BALF samples from BALB/c mice. Notably, the production of some, including MIP-1 α , peaked at both day 0 and day 5.

IL-17 belongs to a family of cytokines consisting of six members: IL-17 (also called IL-17A, the prototype) and IL-17B–F; IL-17E is also known as IL-25. Analysis of cytokines associated with severe asthma in humans has demonstrated elevated levels of IL-17A, implicating activation of the Th17 pathway [25]. Although there was no significant increase in serum IL-17 production at day 3 after RSV inoculation from RSV-infected mice as well as OVA-sensitized/challenged mice in this study, a recent report showed that the production of both IL-17A and IL-5 in lung samples at days 4 and 7 after RSV inoculation was elevated in RSV-infected BALB/c mice [26]. Another report demonstrated that IL-17 production from lung draining lymph nodes was significantly elevated at days 6 and 8 after RSV infection in BALB/c mice [27]. In the same report, RSV restimulation was shown to enhance IL-17 production in samples of cockroach-sensitized RSV-infected mice compared with RSV-infected mice. Furthermore, they also demonstrated that neutralization of IL-17 led to a significant decrease in the exacerbated allergic airway responses, including reduced mucous production, viral load and altered CD8 T-cell marker expression in the mice. Collectively, these observations suggest that the production of MIP-1 α and IL-17 is enhanced by both the antigen-sensitization/challenge and RSV infection in asthma models, leading to airway inflammation.

We found higher levels of serum IL-10 in RSV-infected mice but not in OVA-sensitized/challenged mice with or without RSV infection. These results were comparable with previous reports. For example, RSV infection has been shown to induce expression of IL-10 in human macrophages [28] and in mouse pulmonary T cells [29]. A recent report demonstrated that IL-10 production by CD4⁺ and CD8⁺ T cells in the airways was induced in RSV-infected BALB/c mice at day 8 [30]. Interleukin-10 is an important regulatory cytokine that can mediate a

number of biological activities. However, its role in allergic inflammation is complex [31]. On the one hand, IL-10 has been shown to inhibit IL-5 production, eosinophilic inflammation and mucous production [32, 33], while on the other hand, it might interfere with Th1 cytokine synthesis [31]. In subjects with asthma, both IL-10 mRNA transcripts and IL-10 levels were reduced in BALF [34]. In antigen-sensitized and challenged mice, IL-10 levels both in BALF and serum were undetectable [35].

In this study, we have shown the elevation of IL-12(p40) in BALF from OVA-sensitized/challenged mice with or without RSV infection. In contrast to our results, Barends et al. [36] reported that IL-12 mRNA expression in lung tissue from OVA-allergic BALB/c mice infected with RSV was diminished compared with RSV-infected mice. Systemic administration of IL-12 has been reported to abolish airway hyperresponsiveness, pulmonary eosinophilia and Th2 cytokines in a mouse model of allergic asthma [37]. The cytokine IL-12 plays an essential role in Th1 development and has therefore also been considered as a potential therapy for asthma. IL-12 is a heterodimeric cytokine produced by activated monocytes, macrophages, neutrophils and dendritic cells. Biologically active IL-12 is composed of a covalently linked 35-kDa L chain (p35) and a 40-kDa H chain (p40). The p40 subunit of IL-12 is shared by IL-23, a cytokine with actions similar to but also distinct from IL-12 [38]. Previous reports have demonstrated that IL-12p40 homodimer inhibits IL-12-mediated or IL-23-mediated biological activity by blocking the binding of IL-12 or IL-23 to their respective receptors [39–41], suggesting that IL-12(p40) is a natural antagonist of IL-12 and IL-23. Indeed, IL-12(p40) was shown to reduce IL-12-mediated Th1 responses in vivo [42] and antitumor activity by IL-12 gene therapy [43]. These observations correspond with our results showing elevated IL-12(p40) production in an experimental asthma model.

Finally, in this study, airway responses were relatively limited in mice with RSV alone compared with previous reports [23, 36]; the differences noted in the responses between previous reports and ours may be due to variations in the doses of virus, species of mice used, sensitization protocols, allergens or the intervals between the challenge and measurement period.

In conclusion, our results and previous observations indicate that RSV infection in antigen-sensitized/challenged mice augments the synthesis of cytokines/chemokines including MIP-1 α and IL-17 and airway eosinophil infiltration, all of which are responsible for airway inflammation in asthma.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the contents of this article.

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