

## Influence of respiratory syncytial virus infection on cytokine and inflammatory responses in allergic mice

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### Summary

**Background** Th2 lymphocyte responses are associated with inflammation and disease during allergic responses. Exposure to particular environmental factors during the expression of allergy could result in more pronounced Th2-like immune responses and more severe disease. One factor might be a respiratory virus infection.

**Objective** The aim of our study was to investigate the influence of respiratory syncytial virus (RSV) infection on the expression of ovalbumin (OVA)-induced allergy in BALB/c mice.

**Methods** We determined OVA-specific IgE in serum, cytokine profiles and histopathological lesions in lungs of OVA-allergic mice after RSV infection.

**Results** OVA sensitization and challenge induced OVA-specific IgE in serum, Th2 cytokine mRNA expression, and mononuclear and eosinophilic inflammation in the lungs. RSV inoculation during the challenge period enhanced OVA-induced IL-4 and IL-5 mRNA expression in lung tissue. RSV further enhanced the OVA-induced hypertrophy of mucous cells and eosinophilic infiltration in lung tissue. Surprisingly, RSV infection decreased Th2 cytokine secretion and eosinophilic influx in bronchoalveolar lavage of OVA-allergic mice. Because inactivated RSV did not influence these responses, replication of RSV appeared essential for the modification of OVA-induced Th2 cytokine expression. RSV did not change OVA-specific IgE levels in serum. Furthermore, the RSV-induced IL-12 mRNA expression in lung tissue of OVA-allergic mice was diminished, but IFN- $\gamma$  mRNA expression was not affected.

**Conclusion** RSV infection enhanced particular OVA-induced Th2 cytokine mRNA responses and pulmonary lesions in allergic mice and thus aggravated allergic respiratory disease.

**Keywords** RSV, allergy, ovalbumin, Th1/ Th2 cytokines, eosinophils, IL-4, IL-5, IL-12, IFN- $\gamma$

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### Introduction

Respiratory allergies are characterized by pronounced Th2 lymphocyte responses, including IL-4 and IL-5 cytokine expression, enhanced IgE responses, mast cell degranulation, and influx of activated eosinophils [1–3]. Several authors examined whether respiratory viruses influence the development or expression of respiratory allergy. For example, Rakes et al. [4] examined the prevalence of respiratory viruses in wheezing children and controls. They showed that the majority of childhood wheezing is associated with infection with respiratory syncytial virus (RSV) in infants before 2 years of age, and with rhinovirus in older children. Skoner et al. [5] studied the effects of a rhinovirus infection on immune and inflammatory parameters in allergic and non-allergic persons. Their most important

finding was the increased total serum IgE level in rhinovirus-infected allergic subjects compared with non-infected allergic subjects and rhinovirus-infected non-allergic subjects. Many population studies have been performed in which the relation of RSV infection in early childhood and the development of allergy later in life were investigated. For example, Sigurs et al. showed in a cohort study with matched controls that RSV infection during the first year of life is an important risk factor for the development of asthma and the sensitization to common aeroallergens at the age of 7 years [6,7]. However, controversy exists about the long-term effects of RSV on the development or expression of allergic asthma. Stein et al. failed to confirm the relation between a childhood RSV infection and allergy in teenagers [8]. Unfortunately, nothing is known about the course of an RSV infection in persons who already developed an allergy, or about the influence of RSV infection on the expression of allergy.

A recent study by Peebles et al. [9] showed in mice that an RSV infection during the expression phase of ovalbumin-induced allergy enhanced airway mucus production, increased numbers of lung lymphocytes, and prolonged the period of

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airway hyper-reactivity. These data support the study of Blyth et al. [10], who demonstrated an increased discharge of goblet cell mucin in the airways of allergic mice infected with RSV.

The aim of the present study was to examine whether RSV enhances Th2 cytokine responses during respiratory allergy that could explain the RSV-enhanced allergic response. Therefore, we have determined the influence of an RSV infection on the expression of an OVA-induced respiratory allergy, which is characterized by cytokine responses in the lungs, pulmonary lesions and enhanced serum IgE levels. In this study we demonstrate that RSV infection during OVA-challenge enhanced the OVA-induced Th2 cytokine response in lung tissue but not in bronchoalveolar lavage.

## Methods

### Virus

Human respiratory syncytial virus stock A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The virus was cloned by limiting dilution on HEp-2c cells (ATCC) in 24-well plates. Stock virus was subsequently grown on Hep-2 cells in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK), containing 10% heat-inactivated fetal calf serum (FCS, Hyclone Laboratories Inc., Logan, UT, USA), 2 mM glutamine, 100 IE/mL penicillin and 100 units/mL streptomycin. Cells and media were harvested when *c.* 75% cytopathic effect was observed, sonificated on ice for  $3 \times 5$  s (Vibra cell model VC-40, 40 W, 60% output control. Sonics & Materials Inc., Danburg, CT, USA) and centrifuged for 10 min at 1000 *g*. The supernatant was divided into aliquots and snap frozen at  $-80^{\circ}\text{C}$ . The suspension contained  $10^8$  plaque-forming units (pfu) RSV/mL. Virus was quantified according to Warris et al. [11]. To inactivate RSV, the original stock was divided into small aliquots and UV irradiated (302 nm, dose  $1.5 \times 10^{-2}$   $\mu\text{W}/\text{mm}^2$ , Transilluminator, Ultraviolet Products, San Gabriel, CA, USA) for 45 min [12].

### Animals and experimental design

Female BALB/c mice (RIVM, Bilthoven, the Netherlands) were used at 7 to 9 weeks of age. The institute's committee on animal welfare approved the study. Mice were made allergic to OVA by administration of seven intraperitoneal (i.p.) injections of 0.5 mL OVA (20  $\mu\text{g}/\text{mL}$  saline, no adjuvant), every other day starting at day 0. After 3 weeks mice were challenged by OVA aerosols (2 mg/mL saline) during 5 min on eight consecutive days [13] (Fig. 1).

To examine the influence of RSV infection on OVA-allergy, OVA-allergic mice were inoculated intranasally with  $10^7$  pfu

RSV according to the schedule as outlined in Fig. 1. These mice were compared with OVA-allergic mice sham-inoculated with an uninfected HEp-2c cell lysate (mock), non-allergic mice (sensitized and challenged with saline) and non-allergic mice infected with RSV.

To examine the influence of RSV replication, OVA-allergic mice were inoculated with UV-inactivated RSV. These mice were compared with OVA-allergic mice infected with  $10^7$  pfu RSV.

At different time points after the last OVA-challenge, mice were anaesthetized with barbiturate/chloral hydrate i.p. before they were killed. To examine if RSV replicates in the lungs of OVA-allergic mice (in our experimental setting), one experiment was performed wherein mice were killed at days 4 and 6 post-infection. Lungs were homogenized and the virus titre was determined according to Warris et al. [11].

Each experiment was performed in duplicate and data of one representative experiment are shown.

### Histopathological examination

Lungs were removed, filled intratracheally with formalin and immersed in fixative. Lungs were embedded in paraplast (Monoject, Kildare, Ireland), transverse sections of 5  $\mu\text{m}$  were stained with haematoxylin and eosin. Different lung lesions were scored semiquantitatively from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5).

### Bronchoalveolar lavage

Lungs were lavaged three times using 1 mL of saline ( $37^{\circ}\text{C}$ ) and the BAL was kept on ice until further processing. Supernatant was obtained by centrifugation and stored at  $-80^{\circ}\text{C}$  until cytokines were determined by ELISA. Cytospin preparations were stained with May-Giemsa/Grünwald. In each cytospin at least 400 cells were differentiated into mononuclear cells, neutrophils and eosinophils.

### RNA isolation and RT-PCR

Total cellular RNA was isolated from frozen lung samples using the SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's protocol. cDNA was made by reverse transcriptase (RT) reaction using poly(dT) primers. cDNA was amplified by PCR using specific sense and antisense primers (IFN- $\gamma$ , IL-4, IL-5, IL-12, IL-13 and eotaxin; final concentration 2 ng/mL) [14]. The PCR reactions and analysis were performed as described by Boelen et al. [15]. Every PCR of all samples of one experiment were done in the same reaction and run on the same agarose gel for quantification. All results were corrected to the sample's mRNA content using  $\beta$ -actin mRNA, which was amplified according to the manufacturer's instructions (mouse control amplifier set, Clontech, Palo Alto, CA, USA).

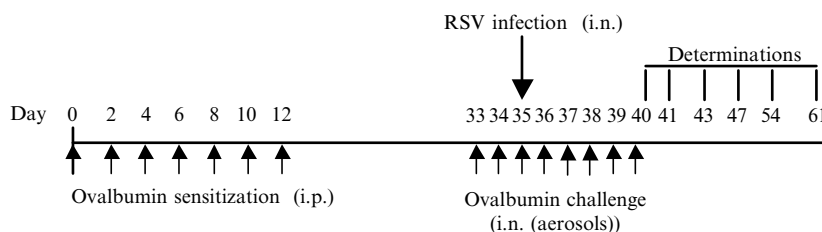


Fig. 1. Study design.

### Cytokine ELISAs

The concentrations of IFN- $\gamma$ , IL-4, IL-5 and IL-13 in the BAL were assessed by ELISA according to the protocol described by Boelen et al. [15]. Coating antibodies were rat antimouse cytokine antibody IgG (IFN- $\gamma$ -IgG<sub>1</sub>, clone R4-6A2; IL-4-IgG<sub>1</sub>, clone 11B11; IL-5-IgG<sub>1</sub>, clone TRFK-5, all derived from Pharmingen, San Diego, CA, USA, and IL-13-IgG<sub>2b</sub>, clone 35213.11, R & D Systems, Minneapolis, MN, USA). Detection antibodies were biotin-conjugated monoclonal rat-anti-mouse cytokine IgG (IFN- $\gamma$ -IgG<sub>1</sub>, clone XMG1.2; IL-4-IgG<sub>1</sub>, clone BVD6-24G2; IL-5-IgG<sub>2a</sub>, clone TRFK-4; all derived from Pharmingen). IL-13 was detected using biotin-conjugated goat anti-mouse IL-13-IgG (R & D Systems). The detection limits of the assays were: IFN- $\gamma$  30 pg/mL; IL-4, 7 pg/mL; IL-5, 30 pg/mL; IL-13, 15 pg/mL. All samples of one experiment were measured within the same assay.

### IgE antibodies in serum

Both total and OVA-specific IgE antibodies were determined in serum using a modified capture ELISA according to the protocol of Van Halteren et al. [16], using rat anti-mouse IgE monoclonal antibody EM-95 [17] as coating antibody. As detecting antibody for total IgE, biotinylated rat-antimouse IgE was used (Pharmingen). OVA-IgE was detected with digoxigenin-coupled ovalbumin. Total IgE concentrations were calculated by interpolation from a standard titration curve with known concentrations of recombinant mouse IgE<sub>3</sub> (Pharmingen). The detection limit of the total IgE assay was 900 pg/mL. OVA-specific IgE levels were expressed as the optical density at 490 nm of a 1/32 diluted serum.

### Data analysis

Comparisons between experimental groups on one single time-point were made using the Student's *t*-test. Comparisons of histological scores were made using the non-parametric Wilcoxon's test. Series of time-points were analysed by analysis of variance (ANOVA) with two grouping factors (time and treatment) using Excel (Excel, Microsoft Corporation, Redmond, WA, USA).

## Results

### Characterization of the OVA-induced allergy

To characterize the OVA-induced allergy we determined total and OVA-specific IgE, eosinophil influx in lungs and bronchoalveolar lavage, hypertrophy of the mucous cells in the bronchiolar epithelium resulting in the production of mucus, and the production of Th2 cytokines. These parameters were assessed 1 day after the last OVA-challenge.

**IgE antibody production in serum** Total serum IgE levels were increased tenfold in OVA-allergic mice compared with non-allergic mice. Furthermore, OVA-specific IgE was only demonstrated in OVA-allergic mice and not in controls.

**Cytokine mRNA expression in lung** We observed approximately threefold increased IL-4, twofold increased IL-5 and fourfold increased IL-13 mRNA expression in the lungs of OVA-allergic mice compared with the background levels in

non-allergic mice. No IFN- $\gamma$  mRNA expression above background level was observed.

**Inflammation of the lungs** We observed an increased influx of eosinophils in BAL of OVA-allergic mice compared with non-allergic mice ( $2.1 \times 10^4$  vs.  $2.9 \times 10^2$  cells/mL,  $P < 0.01$ ). OVA-allergy induced a moderate hypertrophy of the mucous epithelial cells. This hypertrophy was not present in untreated controls.

### The influence of RSV infection on OVA allergy

Subsequently, we examined the influence of RSV infection on the expression of OVA-induced allergy. Therefore, mice were inoculated during the OVA-challenge-phase (Fig. 1).

**The kinetics of RSV infection in OVA-allergic and non-allergic mice** Virus titres in the lungs were determined at days 4 and 6 post-inoculation. No difference in virus recovery from the lungs was observed between the allergic and non-allergic mice. The severity of illness was determined by weight loss. Mice were weighed before and 6 days after RSV inoculation. RSV infection induced severe weight loss but no difference in weight loss was observed between RSV-infected allergic and non-allergic mice (Fig. 2). Nevertheless, these data do not rule out that weight loss at other time-points could have shown differences between those two groups.

**Influence of RSV on histopathological lesions in lungs of OVA-allergic mice** Figure 3 summarizes the pulmonary lesions induced in OVA-allergic mice 1 h, 1 day and 1 week after the last OVA challenge. RSV infection enhanced peribronchiolitis in OVA-allergic mice (mononuclear cells  $P < 0.01$ , eosinophils  $P < 0.001$ ). In addition, RSV infection in OVA-allergic mice enhanced hypertrophy of the bronchiolar mucous epithelial cells and mucus production, compared with mock-inoculated allergic mice ( $P < 0.01$ ). Moderate to marked perivascular infiltrates were predominantly observed in RSV-infected allergic mice ( $P < 0.01$ ).

**Influence of RSV on cytokine mRNA expression in lungs of OVA-allergic mice** We subsequently examined the influence of RSV on the OVA-induced Th2 cytokine responses in lung tissue at several time-points after the last OVA challenge. The results are presented in Fig. 4. RSV enhanced the IL-4 and IL-5 mRNA expression in the lungs of allergic mice compared with mock-inoculated allergic mice ( $P < 0.001$  and  $P < 0.01$ , respectively). In contrast, no significant differences in IL-13 and eotaxin mRNA expression were observed.

**Influence of RSV infection on cytokine content and cell composition of bronchoalveolar lavage in OVA-allergic mice** At day 1 after the last OVA challenge, the IFN- $\gamma$  concentration in BAL was higher in the allergic mice infected with RSV compared with those inoculated with mock. We measured decreased IL-4, IL-5 and IL-13 concentrations in the BAL of RSV-infected allergic mice compared with mock-inoculated allergic mice. However, only the decreased IL-5 concentration was statistically significantly different between those two groups ( $P < 0.05$ ) (Fig. 5).

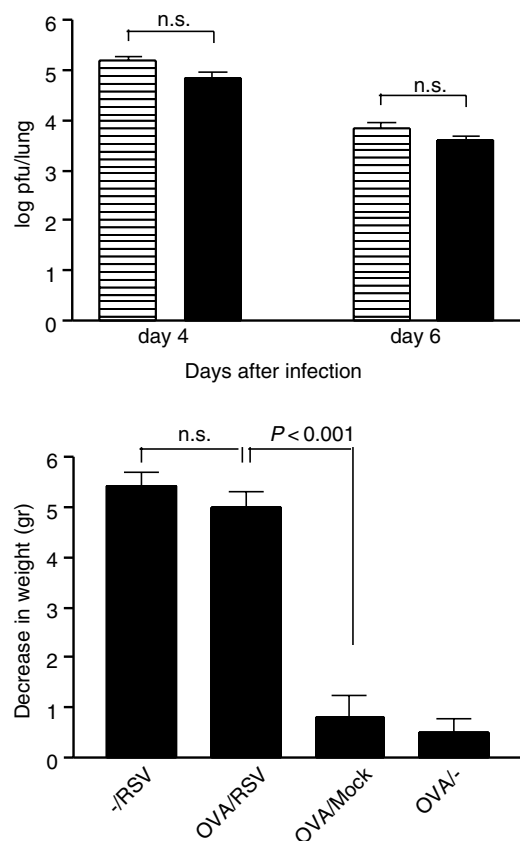


**Fig. 2.** Upper: RSV titres in lung homogenates ( $^{10}\log$  pfu/lung) of OVA-allergic (black bars) and non-allergic (striated bars) mice on 4 and 6 days post-infection. Lower: Weight changes between day 35 and day 41 of OVA-allergic and non-allergic mice with or without RSV infection. The decrease in weight is shown. In both figures mean values  $\pm$  SEM ( $n=6$ ) are depicted. Comparisons between indicated groups were made using Student's *t*-test, n.s. = not significant.

In contrast to the enhanced eosinophil influx in the peri-bronchiolar space in lung tissue, RSV infection decreased the influx of eosinophils in BAL of allergic mice compared with mock-inoculated allergic mice ( $3.7 \times 10^3$  eosinophils/mL vs.  $3.8 \times 10^4$  eosinophils/mL,  $P < 0.001$ ) (Fig. 5).

An increase of neutrophils and lymphocytes in BAL was only observed in RSV-infected mice. No difference was found in neutrophil and lymphocyte numbers in BAL of RSV-infected allergic mice compared with RSV-infected non-allergic mice (Fig. 5). The majority of cells in the BAL of all groups were macrophages and no differences in macrophage numbers were found between the different treatments (data not shown).

**IgE antibody responses in serum** Total IgE production in serum is increased in OVA-allergic mice and OVA-specific IgE is only detected in OVA-allergic mice. In our experiments, RSV infection in allergic mice did not affect both total IgE and OVA-specific IgE levels in serum from mice killed at different time-points during 3 weeks after the last OVA-challenge.

#### *Influence of RSV replication on OVA allergy; comparison of live vs. UV-inactivated RSV*

To determine whether the effects of an RSV infection on OVA-induced allergy were dependent of virus replication in bronchiolar epithelium or were induced by the presence of non-replicating viral antigens, we inoculated OVA-allergic mice with live RSV or with UV-inactivated RSV (UV-RSV).

**Influence of UV-inactivated RSV on cytokine mRNA expression in lungs of allergic mice** We observed no IFN- $\gamma$  mRNA expression in lung tissue of mice inoculated with UV-RSV, while high levels of IFN- $\gamma$  mRNA were detected in RSV-infected allergic and non-allergic mice. As mentioned, RSV enhanced the OVA-induced IL-4 mRNA expression, but UV-RSV did not ( $P < 0.05$ ) (Fig. 6).

**Cytokine and cell composition of bronchoalveolar lavage** In contrast to live RSV, UV-RSV did not induce the production of IFN- $\gamma$  in BAL of both OVA-allergic and non-allergic mice ( $P < 0.001$ ). In addition, while RSV infection diminished IL-4, IL-5 and IL-13 production in BAL of OVA-allergic mice, UV-RSV did not ( $P < 0.01$ ) (Fig. 5). No significant differences were measured between allergic mice inoculated with mock compared with those inoculated with UV-RSV. So, UV-RSV did not influence the OVA-induced Th2 cytokine response in BAL.

In contrast to live RSV, which decreased the influx of eosinophils into BAL, no difference was found in eosinophil influx between allergic mice inoculated with UV-RSV or mock (Fig. 5).

#### *Influence of OVA allergy on cytokine response during RSV infection*

We previously demonstrated that RSV infection in non-allergic mice resulted in a dominant Th1 response [15]. Confirming previous results, we observed no IL-4 and IL-5 mRNA expression in lungs of RSV-infected non-allergic mice. At day 6 post-infection, no difference in IFN- $\gamma$  mRNA expression was observed between RSV-infected allergic and non-allergic mice. However, less expression of IL-12 mRNA ( $P < 0.001$ ) was found in RSV-infected allergic mice compared with RSV-infected non-allergic mice (Fig. 6). Thus OVA allergy diminished RSV-induced IL-12 mRNA expression.

## Discussion

In this study we investigated the influence of RSV infection on a pre-existing ovalbumin-induced allergy in BALB/c mice. OVA sensitization and challenge induced strong Th2 cytokine mRNA expression in the lungs, OVA-specific IgE production in serum, and pulmonary lesions (increased mucus production and eosinophilic influx).

We hypothesized that RSV infection induced more pronounced Th2 cytokine profiles in the lungs of OVA-allergic mice. In our animal model we indeed showed that RSV infection enhanced the OVA allergy-associated cytokine mRNA responses in the lung. IL-4 and IL-5 mRNA expression was specifically increased after RSV infection, and a raised eosinophilic influx in lung tissue and mucus production in the bronchioles was observed.

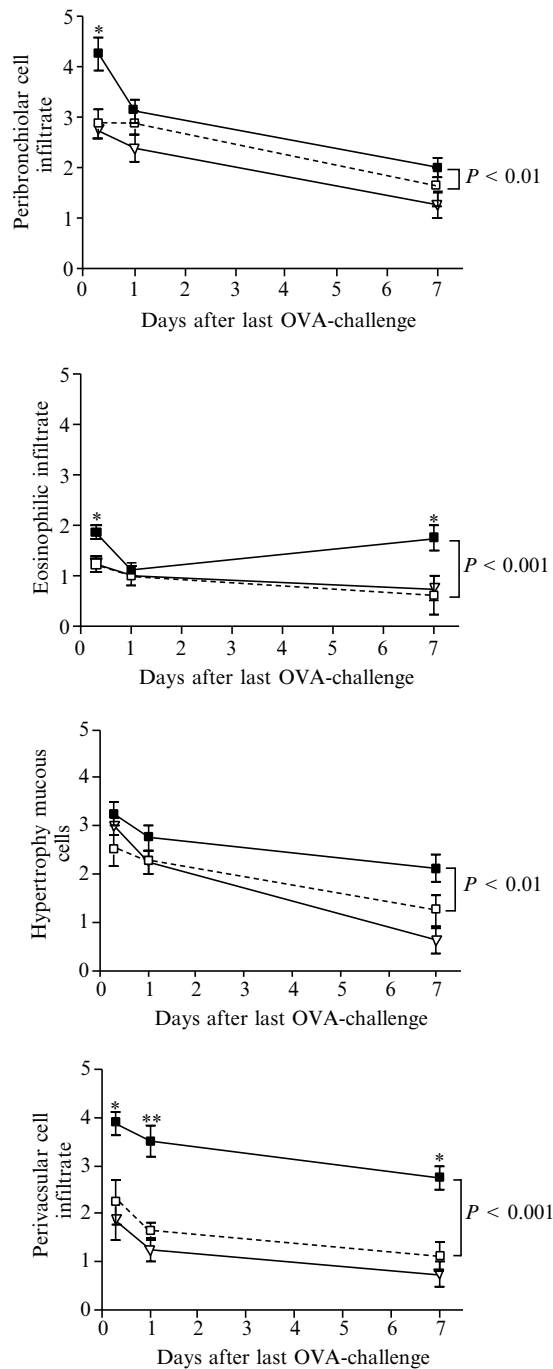


**Fig. 3.** Histopathological changes in lungs of OVA-allergic mice infected with RSV (■-■), inoculated with mock (□-□) or uninfected (▽-▽), determined at 1 h, 1 day and 1 week after the last OVA-challenge. Pulmonary lesions are scored from minimal (1) to severe (5). Scores are depicted as mean value  $\pm$  SEM ( $n=8$ ). Only the statistical differences between OVA/RSV and OVA/mock are shown. Series of time-points were analysed by analysis of variance in accordance with Fisher's least significant difference strategy. Statistical difference on one single time-point between OVA/RSV and OVA/mock is analysed using the non-parametric Wilcoxon's test; differences are indicated by \* $P < 0.05$ , \*\* $P < 0.01$ .

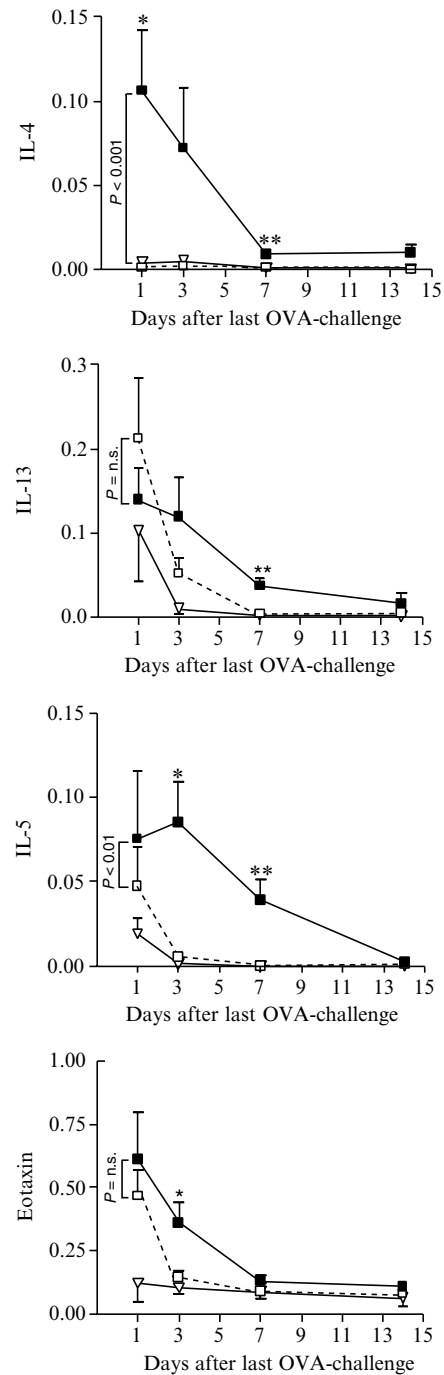


**Fig. 4.** Relative expression (against  $\beta$ -actin) of lung IL-4, IL-5, IL-13 and eotaxin mRNA in OVA-allergic mice infected with RSV (■-■), inoculated with mock (□-□) or uninfected (▽-▽), determined at 1, 3, 7 and 14 days after the last OVA challenge. Mean values  $\pm$  SEM ( $n=6$ ) are depicted. Only statistical differences between OVA/RSV and OVA/mock are shown. Series of time-points were analysed by analysis of variance in accordance with Fisher's least significant difference strategy and  $P$ -values are depicted left in the graphs. Statistical difference on one single time-point between OVA/RSV and OVA/mock is analysed using the Student's  $t$ -test; differences are indicated by \* $P < 0.05$ , \*\* $P < 0.01$ .

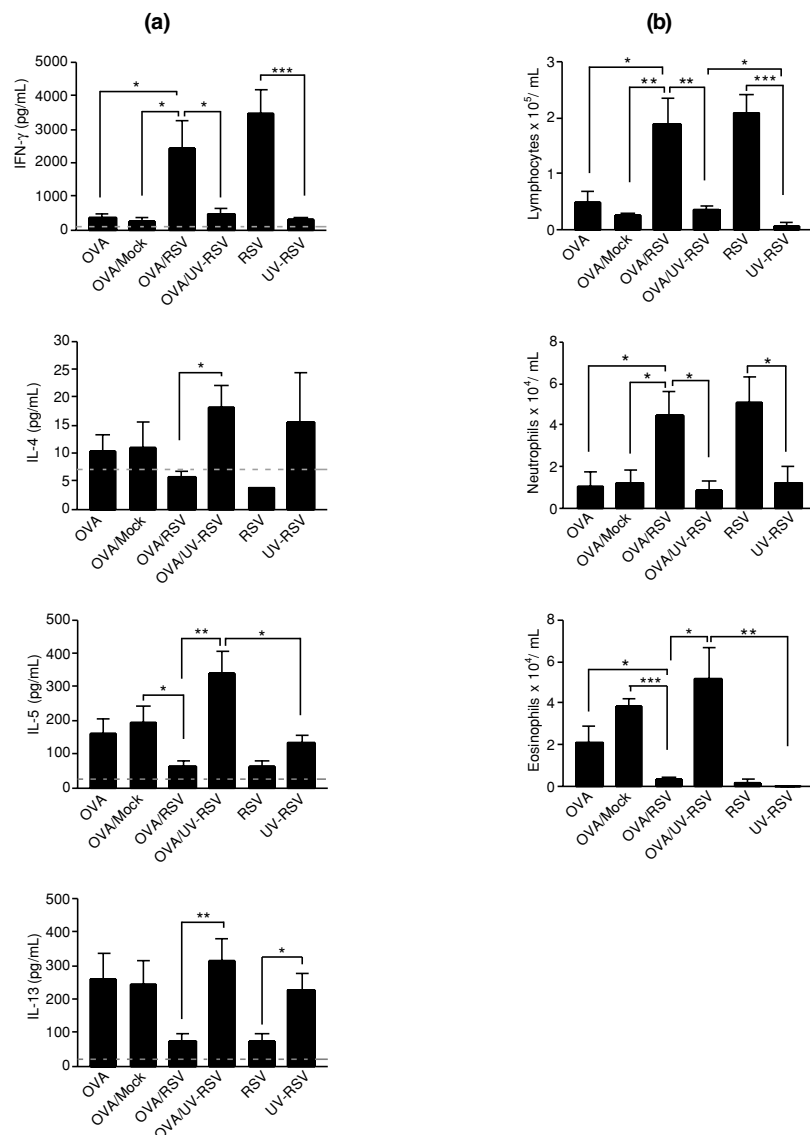


**Fig. 5.** (a): IFN- $\gamma$ , IL-4, IL-5 and IL-13 concentrations in BAL. (b): Composition of cell count from BAL of allergic and non-allergic mice with different treatments (X-axis) determined at 1 day after the last OVA challenge. Mean values  $\pm$  SEM ( $n = 6$ ) are depicted. Statistical differences between indicated individual groups are determined using Student's *t*-test, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Detection limit of the cytokine ELISA assays is presented as a dotted line in the graph.

Several authors have investigated the role of RSV on OVA-induced allergy in mice. In contrast to our experimental protocol, most authors examined the influence of RSV when given before the OVA sensitization period [18,19]. For example, O'Donnell et al. [18] demonstrated an acute collapse in RSV- and influenza virus-infected mice after OVA sensitization during the active phase of viral infection. They observed increased numbers of IL-4-positive splenocytes in RSV-infected mice sensitized with OVA. However, no lung cytokines were determined. In a comparable study, Schwarze et al. [19] observed increased airway hyper-reactivity (AHR) and raised eosinophil influx in the lungs of RSV-infected and OVA-sensitized

mice compared with OVA-sensitized mice only. In the peribronchial lymph nodes they observed increased IL-4 production together with a decrease of IFN- $\gamma$ . This decrease was rather striking because in our experiments we observed raised IFN- $\gamma$  mRNA expression despite increased IL-4.

As far as the authors are aware, only Peebles et al. [9, 20] used the same study protocol in mice as we did, i.e. RSV infection during OVA challenge. They observed increased airway hyper-reactivity when OVA-allergic mice were infected with RSV during the challenge period. However, they did not find increased Th2 cytokine levels in lung tissue, determined both by mRNA expression level and protein concentrations. This is

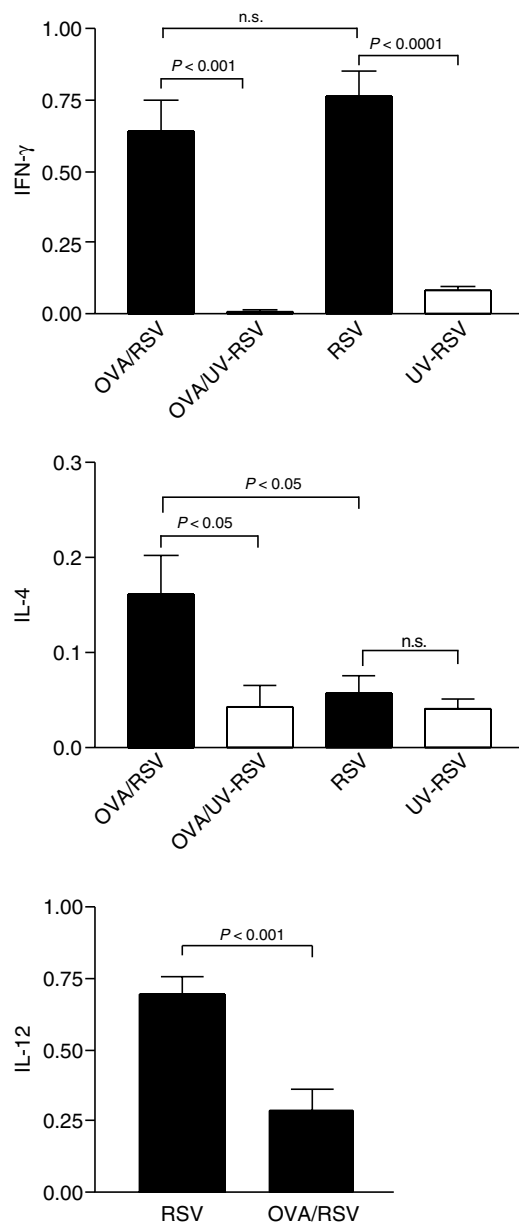


**Fig. 6.** Relative expression (against  $\beta$ -actin) of lung IFN- $\gamma$ , IL-4 and IL-12 mRNA in allergic and non-allergic mice inoculated with live RSV (black bars) or UV-inactivated RSV (open bars), determined at 1 day after the last OVA challenge. Mean values  $\pm$  SEM ( $n = 6-8$ ) are depicted. Comparisons between indicated groups were made using Student's *t*-test.

in contrast to our results because we do see increased Th2 cytokine mRNA expression in lung tissue of RSV infected OVA-allergic mice compared with OVA/mock mice. Possible explanations for the discrepant results could be the different time-points after infection and challenge on which the cytokine levels were determined, and a different method of mRNA detection.

In our experiments IL-4 and IL-5 are the key cytokines affected by RSV in OVA-allergic mice. The enhancement of IL-5 mRNA expression in the lungs correlated with the increased eosinophilic influx in lung tissue. Interestingly,

Schwarze et al. showed in IL-5 deficient mice that RSV-induced IL-5 production during active infection is essential for the influx of eosinophils in lungs and the subsequent development of airway hyper-reactivity (AHR) after OVA challenge [19, 21, 22].

IL-4 is a central cytokine promoting B cells to switch to IgE antibody production. However, we observed that RSV enhanced IL-4 mRNA expression in lung tissue of OVA-allergic mice without increasing allergen-specific or total IgE. A similar observation was made by Schwarze et al. [19], although they studied the influence of an RSV infection on subsequent allergic sensitization. IL-13, independent of IL-4, is crucial in inducing allergic asthma in animal models and is involved in IgE production by B cells [23-25]. In our experiments, RSV did not further increase the OVA-induced IL-13 mRNA expression, which could be a reason for the unaffected serum IgE levels. In a recent study, Tekkanat et al. showed that RSV induces IL-13-dependent airway hyper-reactivity and mucus production in BAL and they suggested that IL-13 and not IL-4 might be an important cytokine in RSV-induced disease [26]. We did not observe increases in the Th2 cytokines IL-4 and IL-5 after primary RSV infection, but the level of IL-13 mRNA in lung tissue was increased (data not shown). However, we did not measure increased IL-13 protein concentration in BAL. IL-13 mRNA expression in OVA-allergic mice, with or without RSV infection, was far more increased than in RSV-infected non-allergic mice.

We and others previously demonstrated that primary RSV infection induced a predominant Th1 cytokine response, characterized by high levels of IFN- $\gamma$  and low levels of IL-4 and IL-5 mRNA expression in the lung [15, 27, 28]. In allergic mice we found a relative decrease in RSV-induced IL-12, but IFN- $\gamma$  expression was unaffected, despite high levels of IL-4. IL-12 is primarily produced by antigen-presenting cells and has a critical role as a regulator of Th1-driven immune responses. The production of IL-12 is inhibited by IL-10, but also IL-4 has a critical role in the regulation of the production of IL-12 [29]. The enhanced IL-4 expression might therefore explain the decrease in IL-12 mRNA expression. The unchanged IFN- $\gamma$  mRNA expression is interesting because of its reported inhibitory function of the Th2 immune response. However, a recent study in atopic children showed that a strong Th2 response might develop despite high levels of IFN- $\gamma$  [30]. In fact, studies in mice indicated that Th1 lymphocytes (producing IFN- $\gamma$ ) may be essential for the migration of Th2 lymphocytes to the airways [31]. It is therefore tempting to speculate that RSV-induced IFN- $\gamma$ -producing cells are essential in the enhancement of the OVA-induced Th2 response.

The RSV-induced enhancement of the OVA-induced Th2 cytokine response and eosinophil influx in lung tissue seem in conflict with the results we obtained in BAL. In BAL we observed decreased IL-4, IL-5 and IL-13 protein concentrations and decreased influx of eosinophils in allergic mice after RSV infection, compared with mock- and UV-RSV-inoculated allergic mice. The airway and lung tissue results, however, may vary because RSV replicates in respiratory epithelium and also in alveolar macrophages. Alveolar macrophages are the majority of cells recovered in BAL and these cells may be activated by RSV [32-34]. These macrophages produce upon stimulation different mediators, among them the cytokines IL-12, IL-1, TNF- $\alpha$  and IFN- $\gamma$ . The synergistic effect of these

cytokines could result in stimulation of the Th1 response and inhibition of the Th2 response. Whatever the mechanism, our results indicate that the outcome of studies on the cytokine and inflammation responses may differ depending on the sites studied.

The pathogenesis of RSV-induced enhancement of allergy is still not clear. Virus-induced immune responses and allergic airway inflammation probably interact to produce altered responses to both virus and allergen [9]. One possible mechanism responsible for the RSV-induced enhancement of OVA allergy could be increased permeability of the respiratory epithelial barrier during RSV infection, which makes it easier for OVA to enter the tissue [35]. In addition, whether other viruses are able to modulate allergic responses similarly to RSV needs to be investigated.

In conclusion, we showed that RSV infection in already allergic mice enhanced the allergy-associated IL-4 and IL-5 cytokine mRNA responses in lung tissue, and increased eosinophilic influx in the lung tissue and mucus production in the bronchioles. However, RSV did not enhance serum IgE levels. Replication of RSV in the lung appears essential for the enhanced allergic responses. Our mice model may allow further mechanistic studies on the influence of RSV on immune and inflammatory responses during childhood allergy.

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