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Effects of 4 months of smoking in mice with ovalbumin-induced airway inflammation

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Summary

Background The effects of smoking on asthma pathogenesis are complex and not well studied. We have shown recently that 3 weeks of smoking attenuates ovalbumin (OVA)-induced airway inflammation in mice and that 4–6 months of smoking induces emphysema in mice without airway inflammation. Effects of combined long-term smoking and OVA exposure have not been investigated so far.

Objective To study whether long-term smoking affects progression of allergic airway inflammation and/or enhances the development of emphysema in mice.

Methods Mice were sensitized to OVA and challenged with saline or OVA aerosols for 6 months. From 2 months onwards, mice were also exposed to air or smoke. Lung tissue was analysed for extent of inflammation, emphysema, remodelling and for cytokine levels, and serum for OVA-specific IgE levels.

Results Chronic OVA exposure of 6 months resulted in a T helper type 2 (Th2)-type inflammation with increased levels of IL-4, IL-5, IL-6 and infiltration of eosinophils, CD4⁺ T cells, macrophages and plasma cells. Smoking induced a Th17-type of airway inflammation, characterized by neutrophils, macrophages, B cells and increased levels of IL-17, IL-6, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor and monocyte chemoattractant protein-1. Concomittant smoking and OVA exposure resulted in inflammation similar to OVA exposure alone. OVA exposure increased IgE levels compared with saline exposure, and smoking did not further increase these levels.

Conclusion We did not find evidence for increased inflammation, IgE levels or emphysema in mice with allergic airway inflammation after 4 months of smoking compared with non-smoking. However, a 4-month exposure to smoke alone did enhance neutrophilic airway inflammation characterized by high pulmonary IL-17 levels. A Th2 inflammatory environment due to OVA exposure may be one explanation as to why no further detrimental effects of smoking on allergic airway inflammation were found.

Keywords active smoking, allergy, asthma, chronic, emphysema, IL-17, inflammation, remodelling, Th17

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Introduction

The effect of active smoking on the pathology of asthma is largely unknown. A few studies have shown that current smokers with asthma have more severe symptoms than asthmatics who have never smoked [1–3] in addition to an accelerated decline in lung function [4]. Other studies have suggested that active smoking may modify the immunological response in asthma with increased neu-

trophils and decreased eosinophils present in sputum and peripheral blood of asthmatic smokers as compared with non-smokers [5, 6]. Because smoking is the main cause of chronic obstructive pulmonary disease (COPD) and asthma is an additional risk factor for developing COPD [7], smoking asthmatics may have an even larger risk of developing COPD [8, 9].

Despite the suggested relationship between active smoking, COPD and asthma, little research has been

carried out into the effects of active smoking on the progression of asthma. Studies in animal models of asthma have shown inconsistent results regarding active smoking and the progression of asthma. We recently demonstrated in a murine model of ovalbumin (OVA)-induced airway inflammation that short-term smoking did not enhance pulmonary inflammation and it even reduced airway eosinophilia [10]. These results were confirmed by Robbins et al., but contradicted by Moerloose et al. [11, 12]. In all mentioned studies, however, mice were only exposed to smoke for a short time, which does not reflect the human situation with usually many years of smoking.

In another study from our hands, we recently showed development of emphysema in normal mice after 4–6 months of smoking [13]. We hypothesized that 4 months of active smoking in mice with pre-existing allergic airway inflammation would worsen the inflammation and accelerate development of emphysema. We therefore studied the degree of airway inflammation, remodelling and emphysema development in mice with or without allergic airway inflammation after 4 months of active smoking.

Material and methods

Animals

Male C57Bl/6j mice (aged 8–10 weeks) were obtained from Harlan (Zeist, the Netherlands) and were held under specific pathogen-free conditions. Experiments, approved by the local Committee on Animal Experimentation, were performed under strict governmental and international guidelines on animal experimentation.

Sensitization and provocation procedures

Mice were sensitized to OVA (Grade V, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) on days 1, 14 and 21 by three intraperitoneal injections of 10 µg OVA emulsified in 1.5 mg aluminium hydroxide (Aluminject, Pierce Chemical, Etten-Leur, the Netherlands) diluted to 200 µL with phosphate-buffered saline (PBS). These mice were subsequently challenged with OVA aerosols (1% in PBS) for 20 min twice a week on consecutive days for 5 months. Control animals were challenged with PBS aerosols.

Smoke exposure procedure

Mice were exposed to mainstream smoke for 5 days a week, two sessions a day during 4 months. As a 'break in' period, two times three puffs of smoke from 2R1 Reference Cigarettes (University of Kentucky) were administered on the first 3 days. Each day thereafter, the number of puffs

was increased with one puff up to two times 24 puffs. For the remainder of the 4 months, two times 24 puffs were given daily, which equals four cigarettes. Control mice were sham-exposed in a separate animal exposure unit, which was placed under similar but smokeless circumstances. These mice were exposed to room air for the same 24 min the smoke exposure took. The smoking machine was checked for the delivery of total particulate matter as described by Griffith and Hancock [14, 15] and calibrated before every smoking session to ensure accurate and standardized smoke delivery. Actual smoke exposure was assessed by measuring carboxyhaemoglobin levels. Smoking mice on average had carboxyhaemoglobin levels of $23.0 \pm 1.7\%$ directly after smoking, whereas control mice had levels of $2.0 \pm 0.5\%$.

Study design

The study design is depicted in Fig. 1a (sensitization, OVA/PBS challenges and smoke exposure protocol) and Fig. 1b (experimental groups). Each of the four study groups consisted of 12 mice. Mice were sensitized to OVA and subsequently either challenged with OVA aerosols (OVA mice) or with PBS aerosols (PBS mice) according to the protocol of Neuhaus-Steinmetz et al. [16] for the induction of chronic allergic airway inflammation. At day 49, when allergic airway inflammation was induced, mice were exposed to either room air (non-smoking or NS) or mainstream smoke (active smoking or S) for 4 months, while OVA and PBS inhalations were continued twice a week. After 4 months, six animals of each group were killed for histological analysis of lung tissue. The remaining six animals of each group were killed for isolation of lung leucocytes and generation of lung homogenates for multiplex ELISA. OVA-specific serum IgE concentrations were assessed on serum acquired by orbita puncture before sensitization (zero time-point), after induction of allergic airway inflammation (month 2 in the entire protocol), and after 1, 2 and 4 months of smoking (months 3, 4 and 6 in the entire protocol).

Lung cell homogenates

Lung tissue was homogenized (20% w/v) in 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl, 0.002% Tween-20 and protease inhibitor (Sigma-Aldrich) and subsequently centrifuged at 12 000 g for 10 min to remove any insoluble material. Supernatants were stored at -80°C until further analysis.

Cytokines

The concentrations of cytokines that are known to be involved in asthma and smoking were measured in lung

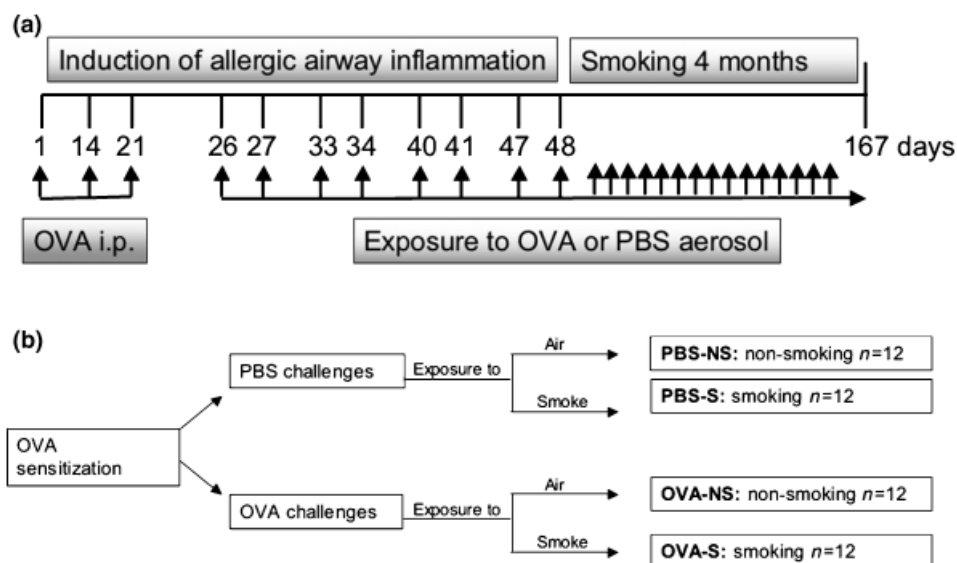


Fig. 1. (a) Experimental design of the study. (b) Experimental groups in the study. OVA, ovalbumin; PBS, phosphate-buffered saline; NS, non-smoking.

homogenates by multiplex ELISA (Lincoplex Systems, St Charles, MO, USA) on a Luminex 100 system using Starstation software (Applied Cytometry Systems, Sheffield, UK). These cytokines included the following: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, KC (murine homologue of IL-8), IL-9, IL-12 (p70), IL-17, IFN- γ , granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1 also known as JE), macrophage inhibitory protein-1 α (MIP-1 α), TNF- α , TGF- β and regulated-on-activation-normal-T-expressed-and-secreted (RANTES). IL-23 was determined in lung tissue homogenates using an IL-23 ELISA according to the instructions of the manufacturer (eBioscience, Malden, the Netherlands).

Detection levels for these cytokines were as follows: IL-1 β = 1.6 pg/mL; IL-2 = 0.8 pg/mL; IL-4 = 0.3 pg/mL; IL-5 = 0.5 pg/mL; IL-6 = 0.7 pg/mL; IL-7 = 3.6 pg/mL; KC = 1.2 pg/mL; IL-9 = 10.5 pg/mL; IL-12 (p70) = 3.7 pg/mL; IL-17 = 1.7 pg/mL; IFN- γ = 0.7 pg/mL; GM-CSF = 4.6 pg/mL; G-CSF = 2.3 pg/mL; MCP-1 = 6.3 pg/mL; MIP-1 α = 2.8 pg/mL; TNF- α = 0.9 pg/mL; TGF- β = 60 pg/mL; RANTES = 0.7 pg/mL and IL-23 = 30 pg/mL.

Lung cell isolation and flow cytometric analysis

Single-cell suspensions were obtained from lungs for flow cytometric analysis as described previously [10]. Cells were quantified using a Coulter Counter Z1 (Coulter, Hialeah, FL, USA).

Expressions of CD3, CD4 and CD25 cell surface markers and GR1 and CD11c cell surface markers on isolated leucocytes were examined with flow cytometry to determine the frequencies of T cell subsets and eosinophils, respectively. All antibodies were obtained from BD Bios-

ciences (Alphen a/d Rijn, the Netherlands) and were conjugated with one of the following commonly used fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (Pe), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC). CD4⁺ T cells were analysed from the lymphocyte gate as defined by forward and side scatter, and positive staining for CD3 and CD4. CD4⁺CD25⁺ effector T cells were defined as lymphocytes positive for CD3, CD4 and CD25. Eosinophils were defined as granulocytes as defined by forward and side scatter and bright positive staining for CD11c and intermediate positive staining for GR1. Cell populations (4×10^4 events) were analysed using an Epics Elite flow cytometer (Coulter Epics, Hialeah, FL, USA) and data analysis was performed using FlowJo (ThreeStar, San Carlos, CA, USA).

Histology

Mast cells were analysed in the airway wall by metachromatic staining of 3 μ m formalin-fixed lung sections with toluidine blue and expressed as the number of positive cells per millimetre airway wall.

Neutrophils and macrophages were identified in 4 μ m cryosections of lung tissue with the rat monoclonal antibodies anti-GR1 (Pharmingen, Alphen a/d Rijn, the Netherlands) and anti-Mac3 (Pharmingen), respectively, using standard immunohistochemical procedures. Both were visualized with 3-amino-9-ethylcarbazole (Sigma-Aldrich). Positive cells were quantified in lung tissue by manual counting of the number of positively stained cells in 14 microscopic fields (magnification $\times 200$). The tissue area in these microscopic fields was subsequently quantified by morphometric analysis and the numbers of cells were expressed per square millimetre.

Inflammatory infiltrates were determined in whole 3 µm sections of formalin-fixed paraffin tissue in a standardized sequence at 10 × 20 magnification. Inflammatory infiltrates were defined as clusters of at least 10 cells. They were quantified in a blinded manner by two independent observers by morphometric analysis using Leica QWin image analysis software (Leica Microsystems BV, Rijswijk, the Netherlands).

B cells, plasma cells and T cells were identified in 4 µm cryosections of lung tissue with monoclonal antibodies against B220 (BD Biosciences), CD138 (Pharmingen) and CD3 (BD Biosciences), respectively, followed by standard immunohistochemical procedures to visualize positively stained cells. B220 and CD138 were visualized with 3-amino-9-ethylcarbazole and CD3 with FastRed (Sigma-Aldrich).

Airway remodelling was defined by goblet cell hyperplasia and increase of collagen III and α -smooth muscle actin (α -SMA) expression in and around airways and blood vessels. All parameters were determined in 3 µm sections of formalin-fixed paraffin tissue. Goblet cells were determined by counting cells staining positive with Periodic Acid Schiff's (PAS), and expressed as the number of PAS-positive cells per millimetre airway. Collagen III and α -SMA were stained with polyclonal goat α -type III collagen (SBA, Birmingham, AL, USA) and monoclonal mouse anti α -SMA (Progen, Heidelberg, Germany), respectively. Both antibodies were further visualized with standard immunohistochemical procedures using FastRed. Collagen III expression was quantified around the airways and blood vessels by morphometric analysis and expressed as volume percentages. α -SMA presence was quantified around the airways by morphometric analysis and expressed as the mean thickness in µm.

Morphometrical evaluation of emphysema

The left lung was inflated and fixed with formalin under a constant pressure of 25 cm H₂O for 24 h. Subsequently, the lung was dissected from the trachea and embedded in paraffin. Emphysema ($n = 6$ per group) was assessed in 3-µm paraffin fixed sections, stained with haematoxylin and eosin. Approximately 25–30 photo microscopic images per tissue section were prepared at 2.5 × 20 magnification using a standardized sequence of image capturing. Images with large vessels, conducting airways or pleura occupying 25% or more of the total image, were not used for the mean linear intercept analysis that was assessed as a measure of alveolar airspace enlargement by two independent individuals in a blinded manner.

Statistical analysis

Homogeneity of variances was checked with Levene's test and data were log-transformed when the variances were

not homogeneously distributed. Multiple linear regression analysis was used for each parameter (except IgE) to establish whether exposures to OVA and smoke interacted. This method disentangles the separate effects of smoking, OVA exposure and their interaction. A significant interaction signifies that the effect of the combination is different (larger or smaller) from the addition of the separate effects of the exposure. We only found significant interactions between OVA exposure and smoking for inflammatory infiltrates in tissue (Fig. 3), IL-17, GM-CSF and KC in lung tissue (Fig. 6). These will be mentioned accordingly in the results section. Mann-Whitney *U*-tests were further used to test the difference between mice exposed to smoking, OVA challenge, or both vs. mice exposed to none. A value of $P < 0.05$ was considered significant. Data analysis was performed with S-Plus software (V6.0, Insightful, Hampshire, UK). Measured values are presented as medians unless stated otherwise.

For the analysis of the longitudinal IgE data, a different approach was adopted. A linear mixed-effects model was fitted to OVA-specific IgE levels in serum. The objective of the analysis was to determine whether OVA-specific IgE changed over time when mice were subjected to OVA challenges, smoke exposure or their combination. Since the animals only started smoking after 2 months, two separate models were used: one to describe the effect of OVA challenges (time 0 until 2 months) and one to describe the effects of OVA challenges and smoking (2 months until 6 months). In these models, OVA challenge and time were included with random effects on intercept and time. However, the variance in the OVA challenged animals was greater and different in nature from the PBS-challenged animals and could not adequately be modelled when analysed together. The PBS-challenged animals were therefore analysed separately in a model containing smoking and time. For the analysis, only mice that had complete data sets were used. Measured values are presented as means $\pm$ the standard error of the mean (SEM) unless stated otherwise.

Data analysis was performed with package nlme version 3.1-68.1 [Jose Pinheiro, Douglas Bates, Saikat Deb-Roy and Deepayan Sarkar (2006). nlme: Linear and non-linear mixed-effects models] in R [version 2.2.1, R Development Core Team (2005). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>].

Results

Smoking increases the number of neutrophils and macrophages

OVA exposure significantly increased numbers of eosinophils, CD4⁺CD25⁺ effector T cells, macrophages and mast

cells in lung tissue in non-smoking and smoking mice (Table 1). OVA exposure had no significant effect on numbers of neutrophils and CD4⁺ T cells in lung tissue.

Smoking, on the other hand, significantly increased the number of neutrophils and macrophages found in lung tissue of PBS-exposed mice. Smoking did not affect numbers of eosinophils, mast cells, and CD4⁺ and CD4⁺CD25⁺ effector T cells.

Linear regression analysis showed no interactions between the effects of OVA exposure and smoking regard-

ing the cell types described in Table 1, meaning that smoking affected the reported cell types in lungs of mice with and without allergic airway inflammation in the same way.

Effects of smoking and ovalbumin-exposure on airway remodelling and the development of emphysema

Four months of smoking did not induce emphysema in either PBS or OVA-exposed mice (Fig. 2a).

Table 1. The effect of OVA-challenge and active smoking on inflammatory cells in lung tissue sections ($n = 6$ per group, mean $\pm$ SEM)

Cell type	PBS		OVA	
	NS	S	NS	S
Eosinophils (% of granulocytes)	3 $\pm$ 1	2 $\pm$ 0.4	16 $\pm$ 5**	18 $\pm$ 6*
CD4 ⁺ T cells (% of lymphocytes)	9.3 $\pm$ 1.2	7.9 $\pm$ 0.5	11.7 $\pm$ 0.7	9.0 $\pm$ 0.6
CD4 ⁺ CD25 ⁺ T cells (% of CD4 ⁺ T cells)	5.4 $\pm$ 0.5	5.8 $\pm$ 0.4	9.3 $\pm$ 1.4*	10.7 $\pm$ 1.4*
Neutrophils (cells/mm ²)	0.1 $\pm$ 0.1	0.7 $\pm$ 0.1**	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1
Macrophages (cells/mm ²)	0.8 $\pm$ 0.2	1.5 $\pm$ 0.5**	1.7 $\pm$ 0.5**	2.4 $\pm$ 0.6**
Mast cells (cells/mm airway)	0 $\pm$ 0	0 $\pm$ 0	0.2 $\pm$ 0.2*	0.2 $\pm$ 0.2**

* $P < 0.05$ vs. PBS-NS.

** $P < 0.01$ vs. PBS-NS.

PBS, phosphate-buffered saline; OVA, ovalbumin; NS, non-smoking; S, smoking; SEM, standard error of the mean

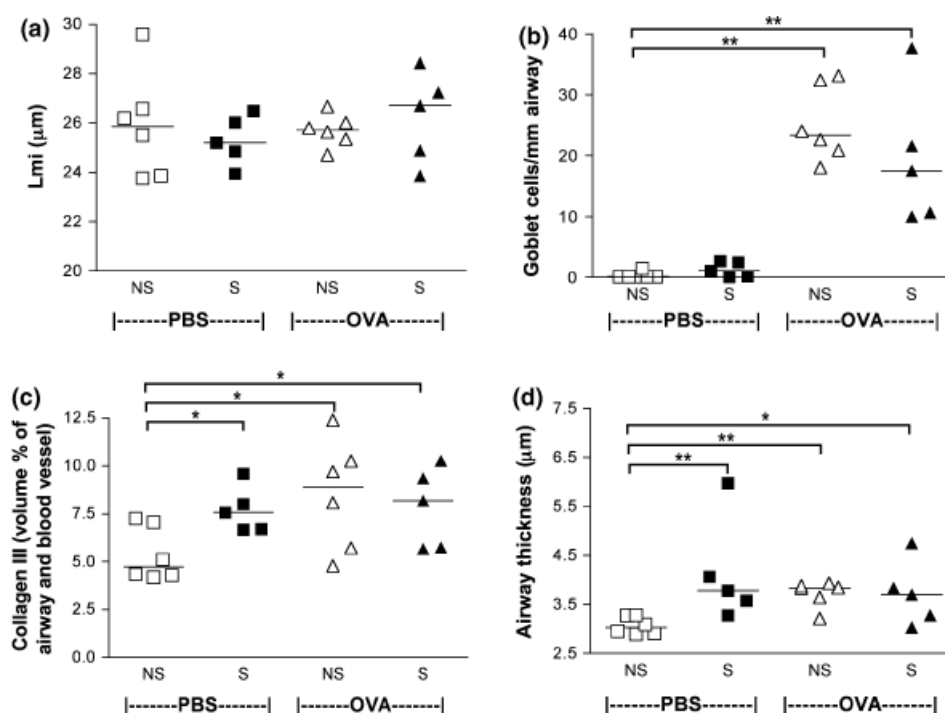


Fig. 2. (a) Ovalbumin (OVA) challenge, smoking or their combination did not cause an increase in emphysema as measured by mean linear intercept (Lmi). (b) OVA challenge increased the number of goblet cells in airways, smoking had no significant effect on goblet cell numbers. (c) OVA challenge, smoking and their combination all increased the deposition of collagen III around airways and blood vessels. (d) OVA challenge, smoking and the combination all increased the deposition of α -smooth muscle actin (α -SMA) around airways leading to an increase in airway thickness. NS, mice exposed to room air for 4 months; S, mice exposed to mainstream smoke for 4 months; PBS, mice exposed to challenges of phosphate-buffered saline for 5 months; OVA, mice exposed to challenges of ovalbumin for 5 months. * $P < 0.05$ vs. PBS-NS, ** $P < 0.01$ vs. PBS-NS. Open squares, PBS-NS; closed squares, PBS-S; open triangles, OVA-NS; closed triangles, OVA-S.

Airway remodelling was defined by goblet cell hyperplasia in the airways, increased α -SMA deposition around the airways and increased deposition of collagen III around the airways and blood vessels. Both smoking and OVA challenge induced features of remodelling (Fig. 2b–d). Linear regression analysis showed no interactions between the effects of OVA exposure and smoking regarding remodelling parameters, meaning that smoking affected mice with and without allergic airway inflammation in the same way.

OVA exposure significantly increased numbers of goblet cells ($P < 0.01$), collagen III ($P < 0.05$) and α -SMA ($P < 0.01$) deposition in non-smokers and smokers. Smoking significantly increased the deposition of collagen III ($P < 0.05$) and α -SMA ($P < 0.01$) in PBS-exposed mice, but had no effect on goblet cell hyperplasia.

Smoking and ovalbumin-exposure induce different types of inflammatory infiltrates

Smoking, OVA exposure and their combination significantly increased volume percentages of inflammatory infiltrates in lung tissue as compared with non-smoking PBS-exposed mice (Fig. 3). Linear regression analysis showed a significant interaction between the effect of OVA exposure and smoking ($P < 0.01$). This interaction is caused by the increase of inflammatory infiltrates after smoking in PBS-exposed mice and the lack of such an (extra) increase in the OVA-exposed mice.

The nature of the infiltrates in lung tissue (Fig. 4) caused by either smoking or OVA exposure was quite different as judged from sections stained for B220 (B cells), CD138 (plasma cells) and CD3 (T cells). In PBS-exposed smoking mice, most infiltrates contained B cells and T cells with some staining for plasma cells. In contrast, in OVA-exposed non-smoking and smoking mice, infiltrates had abundant staining for B cells, plasma cells and T cells.

Smoking increases levels of ovalbumin-specific immunoglobulin E in serum of sensitized mice

Before the start of smoke or air exposure (from time-point 0 to 2 months), OVA challenge significantly increased the serum levels of OVA-specific IgE (Fig. 5). Linear regression showed that OVA-specific IgE levels increased by 0.061 ELISA units [95% confidence interval (CI): 0.04–0.08] per month of exposure more in mice exposed to OVA than in mice exposed to PBS.

During smoke exposure (from 2 to 6 months) only PBS-challenged mice had a significant increase in OVA-specific IgE in time. OVA-specific IgE levels increased 0.0061 ELISA units per month (CI: 0.001–0.011, $P = 0.02$) in smoking PBS mice, without a significant increase in non-smoking PBS mice (increase 0.0001 ELISA units, CI: –0.0035 to 0.0037, $P = 0.9$), the difference between

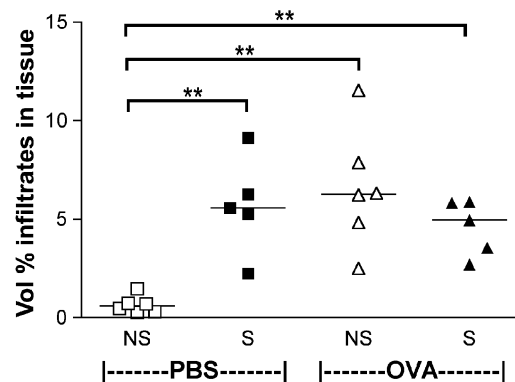


Fig. 3. Smoking and OVA challenge both increased the volume percentage of inflammatory infiltrates (clusters of at least 10 cells) in lung tissue. The combination of smoking and OVA exposure yielded the same results as either of the conditions alone. NS, mice exposed to room air for 4 months; S, mice exposed to mainstream smoke for 4 months; PBS, mice exposed to challenges of phosphate-buffered saline for 5 months; OVA, mice exposed to challenges of ovalbumin for 5 months. $**P < 0.01$. Open squares, PBS-NS; closed squares, PBS-S; open triangles, OVA-NS; closed triangle, OVA-S.

these changes being borderline significant (difference in change: 0.006 ELISA units, CI: –0.0003 to 0.0123, $P = 0.06$).

The already higher levels of OVA-specific IgE in OVA challenged animals were not further affected by smoking: the monthly change in non-smoking mice was 0.0088 ELISA units (CI: –0.01 to 0.027, $P = 0.3$) and in smoking mice –0.004 ELISA units (CI: –0.025 to 0.017, $P = 0.7$). The difference between the change in OVA-specific IgE levels in smoking and non-smoking OVA-mice was not significant (difference in change 0.013 ELISA units, CI: –0.016 to 0.041, $P = 0.4$).

Smoking increases cytokine production in lung tissue related to neutrophil accumulation, B cell proliferation and immunoglobulin E production

After 4 months of smoking and/or exposure to OVA or PBS, we assessed lung tissue concentrations of several cytokines known to be involved in asthma and smoking by multiplex ELISA (Fig. 6).

First of all, the validity of our asthma model was confirmed by the increase in the T helper type 2 (Th2) cytokines IL-4, IL-5 and IL-6 after OVA exposure in both smoking and non-smoking mice. Smoking only increased IL-6 as compared with non-smoking PBS-exposed mice. Of the Th1 cytokines, both IFN- γ (Fig. 6) and IL-12 (data not shown) were not affected by either smoking or OVA exposure.

Smoking increased IL-17, a cytokine exclusively produced by a newly discovered Th subset called Th17 and reported to regulate tissue inflammation [17, 18]. However, it was only increased in smoking PBS-exposed mice. OVA exposure alone or in combination with smoking did

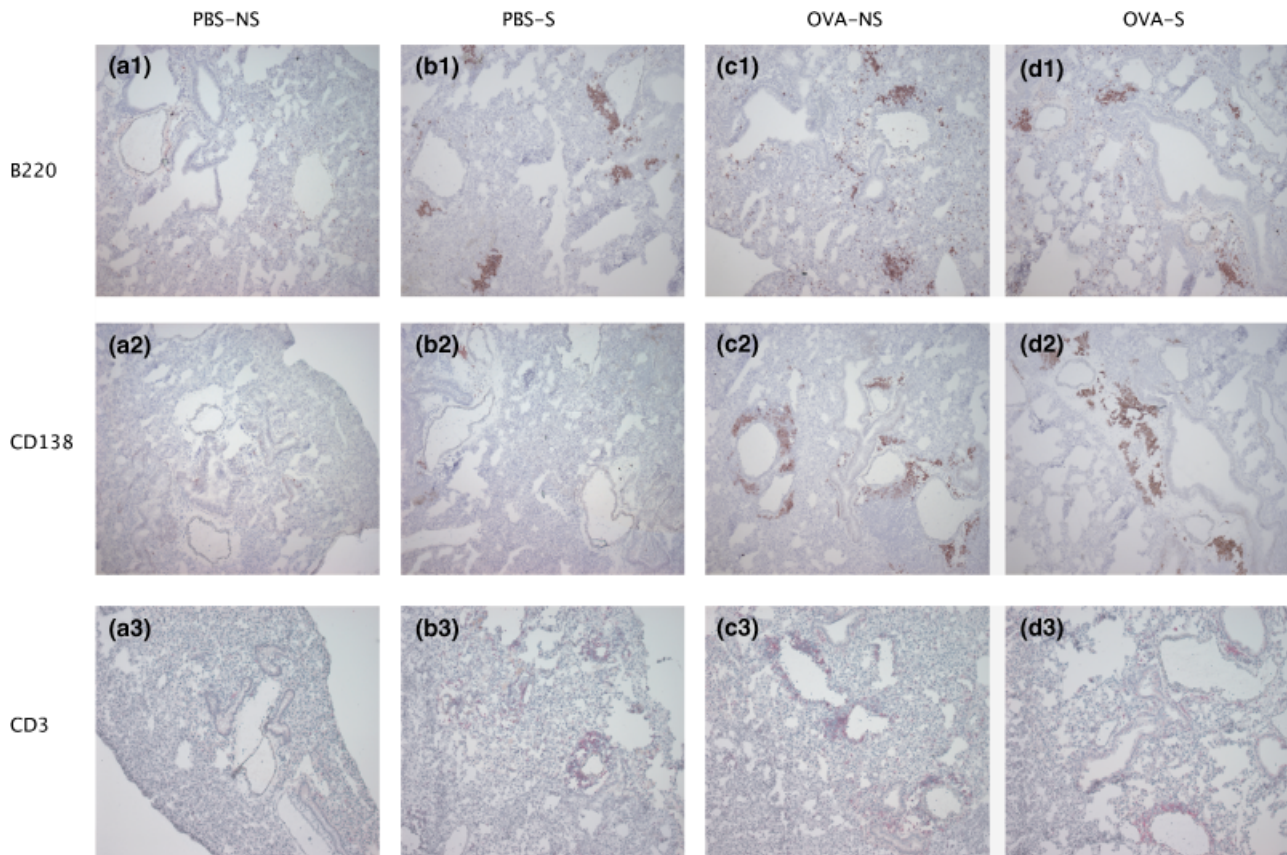


Fig. 4. Mice exposed to PBS challenges and room air did not have inflammatory infiltrates and little staining for B cells (a1), plasma cells (a2) or T cells (a3). In PBS-exposed smoking mice, infiltrates mostly contained B cells (b1) and T cells (b3) with some staining for plasma cells (b2). In contrast, in OVA-exposed non-smoking and smoking mice, infiltrates had abundant staining for B cells (c1 and d1), plasma cells (c2 and d2) and T cells (c3 and d3). NS, mice exposed to room air for 4 months; S, mice exposed to mainstream smoke for 4 months; PBS, mice exposed to challenges of phosphate-buffered saline for 5 months; OVA, mice exposed to challenges of ovalbumin for 5 months. Representative stainings for B220 (B cells), CD138 (plasma cells) and CD3 (T cells) of lung tissue sections. Magnification $\times 25$.

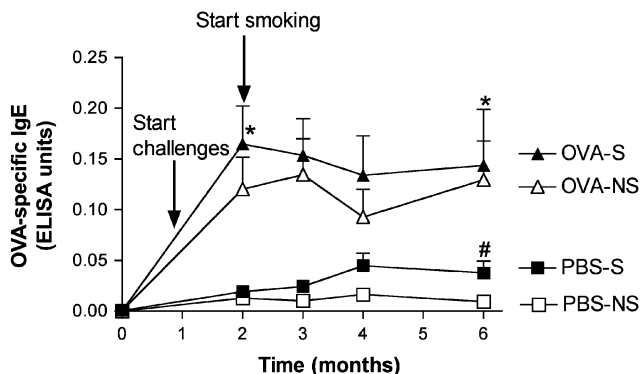


Fig. 5. Exposure to ovalbumin (OVA)-challenges increased OVA-specific IgE as compared with phosphate-buffered saline (PBS)-challenged mice. Smoking increased the levels of OVA-specific IgE in mice sensitized to OVA and challenged with PBS as compared to non-smoking controls. Four months of smoking did not affect IgE levels in OVA-exposed animals. Squares, PBS-exposed mice; triangles, OVA-exposed mice; open symbols, 4-month exposure to room air; closed symbols, 4-month exposure to mainstream smoke. * $P < 0.001$, OVA-exposed animals vs. PBS-exposed animals (independent of smoking). #Significant increase in time ($P < 0.05$) and borderline significant difference between PBS-NS and PBS-S ($P = 0.06$). S, active smoking; NS, non-smoking.

not change IL-17 levels, which therefore generated the significant interaction between smoking and OVA exposure in the linear regression analysis ($P < 0.05$). Cytokines involved in the generation of Th17 and the production of IL-17 are IL-6, TGF- β 1 and IL-23 [19]. The level of IL-6 was increased after smoking, but the levels of TGF- β and IL-23 were not affected by smoking, OVA exposure or their combination.

The cytokines MCP-1, GM-CSF and KC that are associated with IL-17 production were also only significantly increased in smoking PBS-exposed mice (for KC this was a trend, $P = 0.08$). OVA exposure had no effect on the levels of these cytokines. Linear regression analysis showed significant interactions of smoking and OVA exposure for GM-CSF and KC ($P < 0.001$ and < 0.05 , respectively). These interactions were caused by increased levels in the smoking PBS-exposed mice and the absence of these increased levels in OVA-exposed mice.

Smoking, OVA exposure and their combination all increased the levels of G-CSF and MIP-1 α as compared with non-smoking PBS-exposed mice. No differences

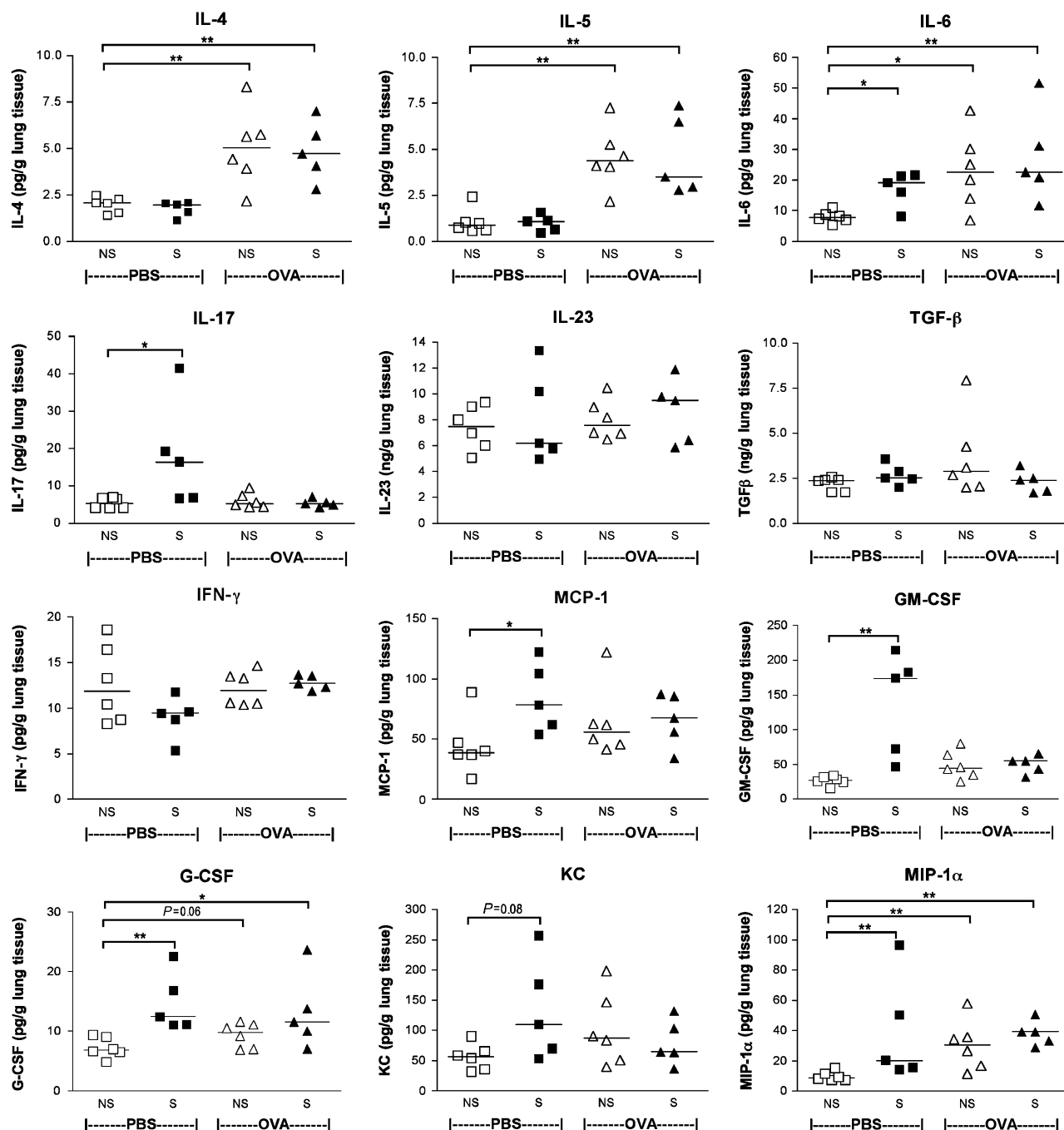


Fig. 6. Cytokine levels in lung tissue of phosphate-buffered saline (PBS)-exposed mice and ovalbumin (OVA)-exposed mice after 4 months of exposure to room air (NS) or smoking (S). No significant differences between the four groups were found for IL-1 β , IL-2, IL-7, IL-9, IL-12 (p70) and RANTES (data not shown). * $P < 0.05$; ** $P < 0.01$. RANTES, regulated-on-activation-normal-T-expressed-and-secreted; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; MIP-1 α , macrophage inhibitory protein-1 α ; MCP-1, monocyte chemoattractant protein-1. Open squares, PBS-NS; closed squares, PBS-S; open triangles, OVA-NS; closed triangle, OVA-S.

between the four groups of mice were found for the levels of IL-1 β , IL-2, IL-7, IL-9 and RANTES (data not shown).

Discussion

This study assessed the effects of long-term smoking on the progression of established asthma and yielded several

interesting findings. First of all, we did not find evidence that smoking aggravates OVA-induced airway inflammation and remodelling or that this combination accelerates the development of emphysema. Smoking also does not further enhance OVA-specific IgE in mice sensitized and challenged with OVA (mice with allergic airway inflammation). Smoking alone, however, induces a Th17-type

airway inflammation with remodelling and also increases OVA-specific IgE levels in mice that have only been sensitized against OVA i.p., but were *not* subsequently challenged. Additionally, we found differences in the nature of the inflammatory infiltrates in lung tissue after smoking and after OVA-induced allergic airway inflammation.

For these studies, we adopted a model of chronic asthma described by Wegmann et al. [20] that resembles many morphological and physiological aspects of human bronchial asthma. Increased levels of Th2 cytokines and eosinophils in lung tissue and persisting OVA-specific IgE, after 5 months of OVA challenges, confirmed the validity of this model. The model further showed increased numbers of mast cells and macrophages and increased tissue remodelling as demonstrated by enhanced deposition of collagen III and α -SMA and goblet cell hyperplasia.

As expected, the nature of the inflammation caused by OVA exposure and by smoking was different. In mice exposed to OVA challenges, a Th2-type of inflammation developed as described above. The inflammatory infiltrates consisted mainly of T cells, B cells and plasma cells, which is compatible with the high levels of Th2 cytokines and OVA-specific IgE found in these animals. IL-4 can efficiently transform infiltrated B cells towards Ig-producing plasma cells, explaining the increase of plasma cells in the lung [21].

As expected, smoking alone caused inflammation that is characterized by increased numbers of tissue neutrophils, macrophages and B cells and increased deposition of collagen III and α -SMA around the airways. The inflammatory infiltrates consisted mainly of B and T cells, confirming our earlier findings in smoking naïve non-sensitized mice [13]. The observed increases in neutrophils and macrophages numbers and collagen deposition after smoking are in line with both clinical studies and other animal studies [22–27].

In addition, smoking in itself caused up-regulation of a different array of cytokines than OVA exposure. An interesting finding is that IL-17 is exclusively increased in lungs of smoking PBS-exposed mice. IL-17 is produced by Th17 cells that develop under the influence of TGF- β 1 and IL-6 and are subsequently expanded from memory T cells with help from IL-23 [17, 18, 28]. Smoking increased IL-6 levels in the lung, which may have helped the development of IL-17-producing T cells. We did not find TGF- β 1 or IL-23 specifically increased after smoking, but this may either be due to a different kinetic track (these levels only represent one point in time) or subtle balances in the ratios of different cytokines. IL-17 mediates inflammation and tissue remodelling by regulating chemokine and metalloproteinases in fibroblasts and epithelial cells [17]. We postulate that smoking favours the generation of effector Th17 cells and that the subsequent release of IL-17 by these cells induces local lung cells to produce

pro-inflammatory cytokines. The elevated levels of G-CSF, GM-CSF, MCP-1 and KC that we found in lungs after smoking support this hypothesis. All these cytokines can be up-regulated by IL-17 or are co-produced by Th17 cells (G-CSF) and are known chemoattractants of neutrophils, macrophages and B cells, cell types we found elevated in smoking mice. Smoking also induced MIP-1 α , which has been shown to attract B cells, an additional explanation as to why we find many B cells after smoking [29, 30].

GM-CSF and IL-17 have both been described to be able to stimulate IgE production by B cells, though not as efficiently as IL-4, which may explain the subtle increase in OVA-specific IgE in smoking PBS-exposed mice [31, 32]. We found that mice that were only subjected to three sensitizations with OVA i.p. and did not receive any OVA challenges, but PBS challenges, had a significant increase in OVA-specific IgE levels after 4 months of smoking. This is specific for OVA-sensitized mice, because we did not find such an increase in experiments with naïve mice subjected to 6 months of smoking (data not shown). If translated to the human situation, this may imply that atopic smokers are at risk of increasing allergen-specific IgE and thereby increasing their risk of developing asthma. Indeed, several epidemiological studies have shown smoking to be a risk factor for the development of asthma [33–36]. From these studies, however, it remains unclear whether atopic smokers are indeed at higher risk of developing asthma, although a small study regarding occupational asthma seems to point in that direction [37]. A recent study in adolescents, however, described a reduced risk of new-onset asthma in atopic smokers [38].

Surprisingly, smoking had no apparent effects on OVA-induced inflammation because the type of airway inflammation was similar in mice only exposed to OVA and mice exposed to both OVA and smoking. This may be explained by Th2 skewing caused by OVA. The development of Th17 cells is blocked by both Th1 and Th2 cytokines IFN- γ and IL-4 [17, 18]. Since smoking started *after* the induction of allergic airway inflammation, IL-4 was already abundantly present and thus inhibits the development of Th17 cells [18, 39]. As a consequence, the effect of OVA exposure would have been dominant over the effect of smoking and subsequently resulted in an inflammatory pattern compatible with OVA-induced inflammation without smoking, as we have found in this study.

Although smoke exposure induced airway inflammation and remodelling, no significant airspace enlargement or emphysema developed when mice with or without airway inflammation were exposed to smoke. In a recently published study, we showed no effect of 2 months, a small effect after 4 months and the largest effect after 6 months of smoke exposure in male C57/bl6 mice as defined by an increase in mean linear intercept in smokers as compared to non-smoking controls, indicating the onset of emphysema [13]. However, in the study presented here, mice

were more slowly exposed to increasing doses of smoke (1 month to full dose instead of 1 week) because we expected that the OVA-exposed animals would react more strongly to smoke exposure than normal animals. Therefore, the total amount of smoke given during these 4 months was less in the current study and probably not sufficiently high to induce emphysema over this time period. The presence of pre-existing asthma, however, did not accelerate this process.

Taken together, we found that 4 months of smoking in sensitized mice without allergic airway inflammation resulted in a different kind of airway inflammation than smoking in mice with allergic airway inflammation. Smoking-induced inflammation in sensitized mice was characterized by IL-17 production and infiltration of neutrophils and macrophages, whereas smoking in mice with allergic airway inflammation did not change the allergic inflammation characterized by the production of Th2 cytokines and infiltration of T cells, macrophages and plasma cells. Smoking in mice with allergic airway inflammation did not accelerate emphysema development or remodelling. The finding that long-term smoking induces IL-17 production, but not in the context of an ongoing Th2 inflammation deserves further attention as a possible mechanism for the lack of smoking-related effects in airway inflammation.

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