

Short-Term Smoke Exposure Attenuates Ovalbumin-Induced Airway Inflammation in Allergic Mice

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Little is known about effects of smoking on airway inflammation in asthma. We tested the hypothesis that smoking enhances established airway inflammation in a mouse model of allergic asthma. C57Bl/6j mice were sensitized to ovalbumin (OVA) and challenged with OVA (OVA-mice) or sham-sensitized to phosphate-buffered saline (PBS) and challenged with PBS aerosols (PBS-mice) for 7 wk. At 4 wk, mice were additionally exposed to air (nonsmoking controls) or mainstream smoke for 3 wk. Using whole body plethysmography, we found OVA-induced bronchoconstriction to be significantly inhibited in smoking OVA-mice as compared with nonsmoking OVA-mice ($1 \pm 2\%$ increase versus $22 \pm 6\%$ increase in enhanced pause, respectively). Smoking did not change airway hyperresponsiveness (AHR) to methacholine in PBS-mice, yet significantly attenuated AHR in OVA-mice 24 h after OVA challenge as compared with nonsmoking mice. This was accompanied by reduced eosinophil numbers in lung lavage fluid and tissue of smoking OVA-mice compared with nonsmoking OVA-mice. In contrast to our hypothesis, short-term smoking reduced responsiveness to OVA and methacholine in OVA-mice and decreased airway inflammation when compared with nonsmoking mice. This effect of smoking may be different for long-term smoking, in which remodeling effects of smoking can be expected to interrelate with remodeling changes caused by asthmatic disease.

More than 30% of the Dutch population smokes, and this percentage is only marginally decreasing (year 2001 figures by NIPO, The Netherlands). The same figures can be found in many countries, and despite well-known negative effects of smoking and many governmental campaigns, alarming numbers of young people still start smoking. Unfortunately, this number is the same for young people with and without asthma (1). The question therefore arises: what are the effects of smoking on preexisting asthma? Despite the obvious relevance, little is known in this respect. It is clear that cigarette smoke exposure *in utero* and/or postnatally increases the risk of developing asthma (2). Furthermore, epidemiologic data have suggested that smokers with asthma experience a more rapid lung function decline than nonsmokers with asthma as well as smokers without asthma (3), suggesting an interaction between smoking and asthmatic airway inflammation. However, the underlying mechanisms of these observations have not been identified. Chalmers and coworkers showed that smoking was associated with a neutrophilic inflammation in both individuals with asthma and those without

asthma, but that it did not change airway hyperresponsiveness (AHR) in those with asthma (4). These data were generated from sputum samples and give little information about the allergic inflammation in the lung itself.

To investigate the acute effects of smoking on established asthma we have used a mouse model of chronic allergic airway inflammation. We tested the hypothesis that smoking enhances established airway inflammation, thereby adversely affecting lung function and AHR. After induction of an asthma phenotype with ovalbumin (OVA), mice were subjected to 3 wk of smoking. The effects of this short period of smoke exposure on AHR and the response to an OVA challenge were assessed and airway inflammation was studied in detail in lung tissue, lung cell isolates, and bronchoalveolar lavage (BAL) fluid.

Materials and Methods

Animals

Male C57Bl/6j mice (aged 8–10 wk) were obtained from Harlan (Zeist, The Netherlands) and were held under specific pathogen-free conditions. The 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 μ g OVA emulsified in 1.5 mg aluminum hydroxide (Aluminject; Pierce Chemical, Etten-Leur, The Netherlands) diluted to 200 μ l 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 7 wk. The aerosol was delivered to a perspex exposure chamber (9 liters) by a De Vilbiss nebulizer (type 646; De Vilbiss, Somerset, PA) driven by an airflow of 40 liters/min providing aerosol with an output of 0.33 ml/min.

Control animals received intraperitoneal injections of aluminum hydroxide diluted with PBS and were subsequently challenged with PBS aerosol.

Smoke Exposure Procedure

The smoke exposure system of the Tobacco and Health Research Institute of the University of Kentucky was used for the nose-only exposure of mice to mainstream smoke, which is similar to active smoking (5). The system was set up according to the instructions of the manufacturer for generation of mainstream smoke.

For 3 wk, mice were exposed to mainstream smoke for 5 d/wk, two sessions per day. On the first 2 d, 2 times 4 puffs of smoke from 2R1 Reference Cigarettes (University of Kentucky) were administered. Two times 6, 8, 10, 12, 15, 18, 21, and 24 puffs were given on subsequent days during a "break in" period. For the remainder of the 3 wk, two times 24 puffs were given daily, which equals 4 cigarettes. Control mice were sham-exposed in a separate animal exposure unit, which was placed under equal but smokeless circumstances. These mice were exposed to room air for the same 24 min the smoke exposure takes.

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Abbreviations: airway hyperresponsiveness, AHR; bronchoalveolar lavage, BAL; carbon monoxide, CO; enzyme-linked immunosorbent assay, ELISA; interleukin, IL; methacholine, MCh; ovalbumin, OVA; phosphate-buffered saline, PBS; enhanced pause, Penh.

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Study Design

The study design is depicted in Figures 1A (experimental groups) and 1B (sensitization, OVA/PBS challenges, smoke exposure protocol, and tests of hyperresponsiveness and OVA/PBS-induced bronchoconstriction). Each of the six study groups consisted of eight mice. Mice were either sensitized to OVA and subsequently challenged with OVA aerosols, or sham-sensitized with PBS and subsequently challenged with PBS aerosols according to the protocol of Neuhaus-Steinmetz and colleagues (6) for the induction of chronic allergic airway inflammation. At Day 49, when allergic airway inflammation was induced, mice were exposed to either room air (nonsmoking or NS) or mainstream smoke (smoking or S) for 3 wk, whereas OVA and PBS inhalations were continued twice a week. Acute airway obstruction after OVA/PBS challenge and AHR were measured after 3 wk of smoke exposure on Days 68 and 70, respectively (for details *see below*). On Day 71, five out of eight animals of each group were killed for BAL fluid and isolation of inflammatory cells from lung tissue. The remaining three animals of each group were killed for histologic analysis of lung tissue. Total and OVA-specific serum IgE concentrations were assessed on Days 1, 36 (Week 6), 43 (Week 7), and 71 (Week 11).

Assessment of Acute Airway Obstruction and Hyperresponsiveness

Acute airway obstruction after OVA or PBS challenge, and AHR, were assessed as changes in enhanced pause (Penh) in conscious, spontaneously breathing animals using a whole-body plethysmography system (Buxco Electronics, Petersfield, UK) as described previously (7). Briefly, mice were placed in individual chambers and allowed to settle for 5–10 min. For the measurement of OVA/PBS-induced bronchoconstriction, baseline Penh readings were taken for 10 min and averaged. The animals were then subjected to an aerosol (OVA or PBS) for 20 min. Immediately thereafter, post-aerosol Penh readings were taken for 30 min and averaged.

To assess AHR, mice were challenged with aerosolized PBS or methacholine (MCh, 2–64 mg/ml; Sigma-Aldrich Chemie BV) for 2 min and readings were taken and averaged for 3 min following each nebulization. From these dose–response curves the provocative concentration causing a 300% increase of the baseline Penh (PC300) was calculated using GraphPad Prism software (V3.1; GraphPad Software, Inc., San Diego, CA). In those cases in which the 300% increase was not reached with the highest concentration of 64 mg/ml MCh, a provocative concentration of 128 mg/ml was assigned.

Measurement of Serum IgE (Total and OVA-Specific)

Total serum IgE was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen, San Diego, CA) following the manufacturer's protocol. OVA-specific IgE was measured as described previously for rats (8) with some minor modifications. Wells were coated

with anti-mouse IgE (Pharmingen) as described in the protocol for the total IgE assay and the levels of OVA-specific IgE were expressed in arbitrary ELISA units, where 1 ELISA unit equals the optical density of the sample divided by the optical density of standard pooled mouse serum.

BAL

BAL was performed on five out of eight anesthetized mice of each group on Day 71, 48 h after the last OVA/PBS challenge and 24 h after the last cigarette. Two 0.5-ml lavage aliquots with cold sterile PBS were pooled and total viable cell counts were determined manually using trypan blue exclusion. After centrifugation at $500 \times g$ for 30 min at 4°C, supernatants were stored at –80°C for analysis of cytokine content and the cells were resuspended in Dulbecco's modified Eagle medium (BioWhittaker Europe, Verviers, Belgium) for preparation of cytoplasts. These were prepared for each sample by use of a cytospin centrifuge (Cytospin 2; Shandon, Pittsburgh, PA): 5 min at $100 \times g$ of 100 μ l cell suspension containing ~ 50,000 cells.

Cytokines

Concentrations of interleukin (IL)-4, IL-5, and interferon- γ in BAL fluid were measured using ELISA kits from Pharmingen.

Cell Preparation and Lung Digestion

Single-cell suspensions were obtained from lungs for flow cytometric analysis and preparation of cytoplasts as described previously (9). Cells were quantified using a Coulter Counter Z1 (Coulter, Hialeah, FL). Cytoplasts of the lung cell isolates were prepared as described for the BAL.

Flow Cytometric Analysis

Expression of CD3, CD4, CD8, and CD25 cell surface markers on isolated leukocytes were examined with four-color flow cytometry to determine the frequencies of T cell subsets. Isolated lung tissue leukocytes were stained with a combination of directly conjugated antibodies directed against these cell surface markers as described previously (9). All antibodies were obtained from Pharmingen and were conjugated with one of the following commonly used fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (Pe), peridinin chlorophyll protein (PerCP), and allophycocyanin (APC). Frequencies of T cell subsets were based on the label combinations: CD3-PerCP, CD4-PE, CD8-APC, and CD25-FITC. Cell populations (4×10^4 events) were analyzed using an Epics Elite flow cytometer (Coulter) and data analysis was performed using FlowJo (TreeStar, San Carlos, CA).

Assessment of Eosinophils, Neutrophils, and Macrophages

Eosinophils were determined in 4- μ m sections of frozen lung tissue and in cytoplasts of BAL fluid or lung cell isolates by staining for cyanide

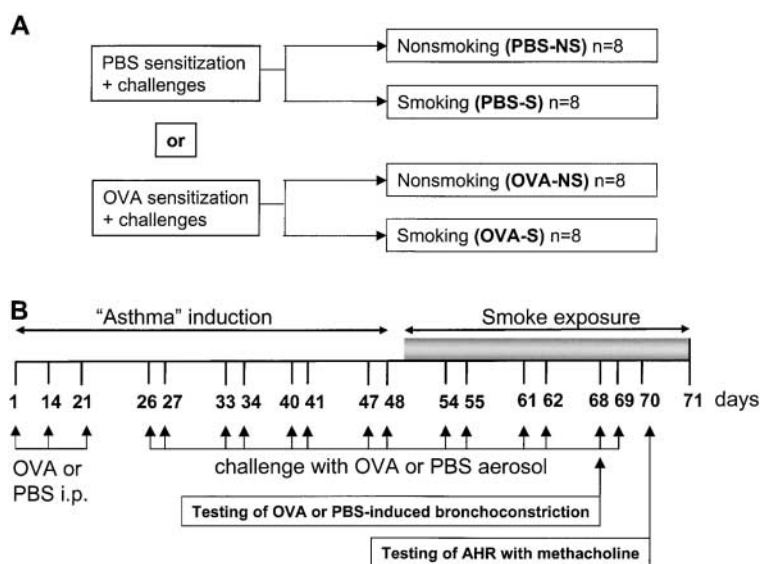


Figure 1. (A) Experimental groups in the study. (B) Experimental design of the study.

resistant endogenous peroxidase activity with diaminobenzidine (Sigma). Eosinophils in lung tissue were quantified by morphometric analysis and expressed as volume percentages (9). To determine the percentage of eosinophils in cytopots of BAL fluid or lung cell isolates, the number of peroxidase-positive cells was counted in a total of 500 cells by two independent observers.

Neutrophils and macrophages were identified with the rat monoclonal antibodies anti-GR1 (Pharmingen) and anti-Mac3 (Pharmingen), respectively. Because of extensive presence of endogenous peroxidase in eosinophils, an alkaline phosphatase-labeled secondary antibody in combination with the chromagen Fast Red TR (Sigma) was used for the detection of positively stained cells. As detection of the Fast Red label is less sensitive in morphometric analysis, these 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 millimeter.

Statistical Analysis

Measured values are presented as means $\pm$ SEM unless stated otherwise. Statistical comparisons were made using ANOVA with Tukey and Sidak multiple comparison tests. A value of $P < 0.05$ was considered significant. Data analysis was performed with S-Plus software (V6.0; Insightful, Hampshire, UK)

Results

Smoking Attenuated OVA-Induced Bronchoconstriction and AHR

Baseline Penh values were not significantly affected by allergic sensitization or smoking (Table 1). OVA sensitization followed by OVA challenges did, however, result in a small but significant increase in airway obstruction (depicted as percentage increase in Penh from baseline) in nonsmoking mice challenged at Day 68 as compared with nonsmoking PBS-challenged controls (Figure 2, $P < 0.001$). Although smoking had no effect on Penh in the PBS-challenged animals, it significantly inhibited bronchoconstriction as present in OVA-challenged mice ($P < 0.05$).

Additionally, smoking significantly decreased the responsiveness to MCh in OVA-challenged animals when compared with the nonsmoking OVA-challenged animals (Figure 3B, Table 2). PC300 values went up from 19.6 ± 2.3 mg/ml for nonsmoking mice to 67.3 ± 18.2 mg/ml for smokers ($P < 0.01$). Smoking had no effect on AHR in PBS-challenged animals (Figure 3A).

No Effect of Smoking on Total or OVA-Specific Serum IgE

Levels of both total IgE and OVA-specific IgE increased significantly at all test times in mice challenged with OVA as compared with the mice challenged with PBS (Figure 4, $P < 0.001$). Smoking had no effect on total or OVA-specific IgE levels.

No Detectable Levels of Cytokines in BAL Fluid

The levels of IL-4, IL-5, and interferon- γ in BAL fluid were below the detection limit of the ELISA kits used. The effects of OVA exposure and smoking on these cytokines can therefore not be determined.

TABLE 1. Baseline Penh values before OVA or PBS challenge ($n = 8$, mean $\pm$ SEM)

	PBS-Challenged Mice, Baseline Penh	OVA-Challenged Mice, Baseline Penh
Nonsmokers	0.63 ± 0.03	0.72 ± 0.04
Smokers	0.69 ± 0.02	0.79 ± 0.03

Definition of abbreviations: OVA, ovalbumin; PBS, phosphate-buffered saline; Penh, enhanced pause.

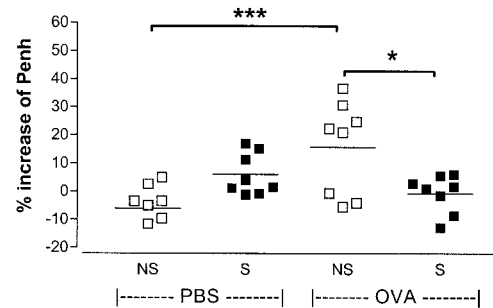


Figure 2. Bronchoconstriction in mice after challenge with PBS (left) or OVA (right) 48 h after either being exposed to room air (NS, open squares) or after smoking for 3 wk (S, filled squares). Results are expressed as the percentage increase of Penh compared with baseline Penh. * $P < 0.05$, *** $P < 0.001$.

Smoking Reduced the Percentage of Eosinophils in BAL Fluid

OVA sensitization followed by OVA challenges slightly increased the total number of cells in BAL fluid of nonsmoking mice as compared with PBS-treated controls (14.6 ± 2.5 versus $12.7 \pm 2.6 \times 10^4$ cells/ml). This increase can mostly be explained by the large increase in the number of eosinophils in BAL fluid after OVA treatment (Figure 5A). Smoking had no effect on the total cell number in BAL fluid ($14.3 \pm 2.0 \times 10^4$ cells/ml), but it did significantly decrease the percentage of eosinophils in BAL fluid of OVA-challenged mice ($26.6 \pm 3.4\%$ for nonsmoking OVA-mice versus $9.7 \pm 3.5\%$ for smoking OVA-mice, $P < 0.05$).

The numbers of lung tissue eosinophils, as assessed in cytopots of lung cell isolates (Figure 5B) and cryostat sections of lung tissue (Figure 5C), were also significantly increased in OVA-challenged animals as compared with PBS-challenged controls.

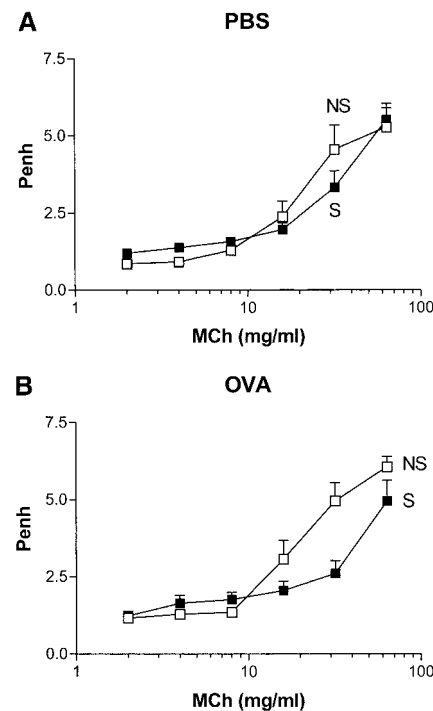


Figure 3. Responsiveness to MCh of mice treated with PBS (A) or OVA (B), 3 h after being exposed to room air (NS, open squares) or after smoking (S, filled squares).

TABLE 2. PC300 calculated from MCh challenge tests ($n = 8$, mean $\pm$ SEM)

	PBS-challenged mice	OVA-challenged mice
Nonsmokers	16.1 $\pm$ 1.2	19.6 $\pm$ 2.3
Smokers	25.4 $\pm$ 2.2	67.3 $\pm$ 18.2*

Definition of abbreviations: MCh, methacholine; OVA, ovalbumin; PBS, phosphate-buffered saline.
* $P < 0.01$ OVA-S versus OVA-NS.

In this case, smoking seemed to have no effect on the number of tissue eosinophils in OVA-challenged mice.

Smoking Downregulated the Number of CD4⁺ T Cells and Activated CD8⁺ T Cells

Sensitization against and challenge with OVA of nonsmoking mice resulted in significantly increased percentages of CD4⁺ T cells and activated CD4⁺ T cells (CD25⁺) in lung tissue ($P < 0.001$) as compared with nonsmoking PBS-control mice (Figures 6A and 6B). Smoking did not inhibit this increase in CD4⁺ T cells significantly, but a trend was observed. Additionally, smoking did not change the percentage of activated CD4⁺ T cells in lungs of OVA- or PBS-challenged animals, or influence T cell percentages in PBS-challenged animals. In contrast, percentages of CD8⁺ T cells were not affected by OVA challenge alone or in combination with smoking (Figure 6C). However, as shown in Figure 6D, OVA treatment did increase the percentage of activated CD8⁺ T cells as compared with nonsmoking PBS controls ($P < 0.05$). Smoking inhibited this increase of activated CD8⁺ T cells in OVA-mice, but it did not affect the percentage of activated CD8⁺ T cells in control PBS-mice.

Effect of Smoking on Numbers of Neutrophils and Macrophages

OVA challenge induced a significant increase in macrophages in lung tissue of nonsmoking animals as compared with PBS

challenge (Table 3). This increase was significantly inhibited in the smoking OVA-mice. The number of macrophages, however, was still significantly higher than in nonsmoking PBS-animals. Smoking also caused a significant increase in macrophages in lung tissue of PBS-mice.

The number of neutrophils in lung tissue was not affected by OVA challenge alone or in combination with smoking. Smoking did increase the number of neutrophils in nonallergic animals.

Discussion

In the present study we aimed at investigating the *short-term* effects of smoking on allergic airway inflammation in a mouse model of asthma. The harmful effects of *long-term* smoking are well known, and include respiratory disorders culminating in the development of chronic obstructive pulmonary disease, cardiovascular hazards, and many types of cancer (3, 10–12). Far less is known about the effects of short-term smoke exposure on the lungs, especially in lungs of patients already afflicted by an allergic inflammation.

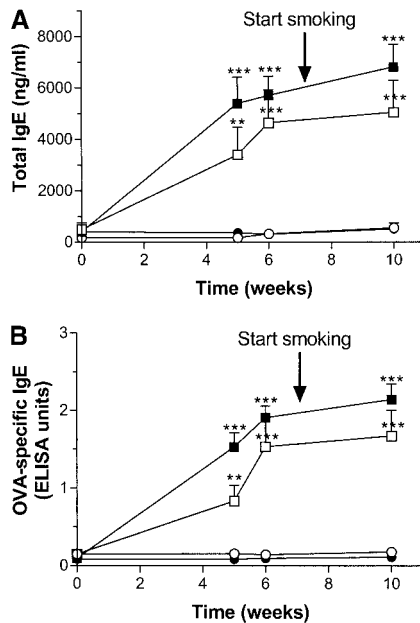


Figure 4. Total serum IgE (A) and OVA-specific serum IgE (B) in mice 0, 5, 6, and 10 wk after sensitization with OVA (squares) or PBS (circles). Mice were either exposed to room air (open symbols) for 3 wk or had smoked (filled symbols) for 3 wk. ** $P < 0.01$, *** $P < 0.001$ versus nonsmoking PBS group.

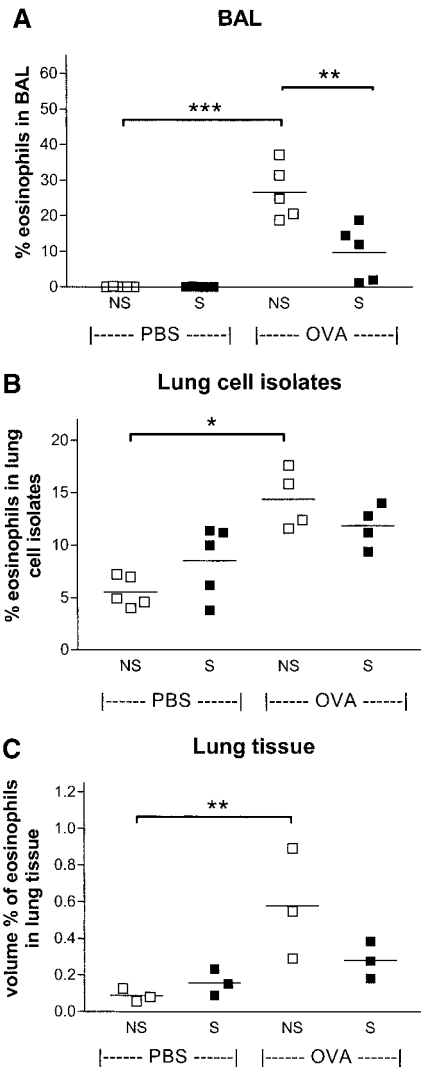


Figure 5. Eosinophils, expressed as percentages in BAL fluid (A) and lung cell isolates (B) and expressed as volume percentages in frozen lung sections (C) in mice treated with PBS (left) or OVA (right) after being exposed to room air (NS, open squares) or after smoking (S, filled squares). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

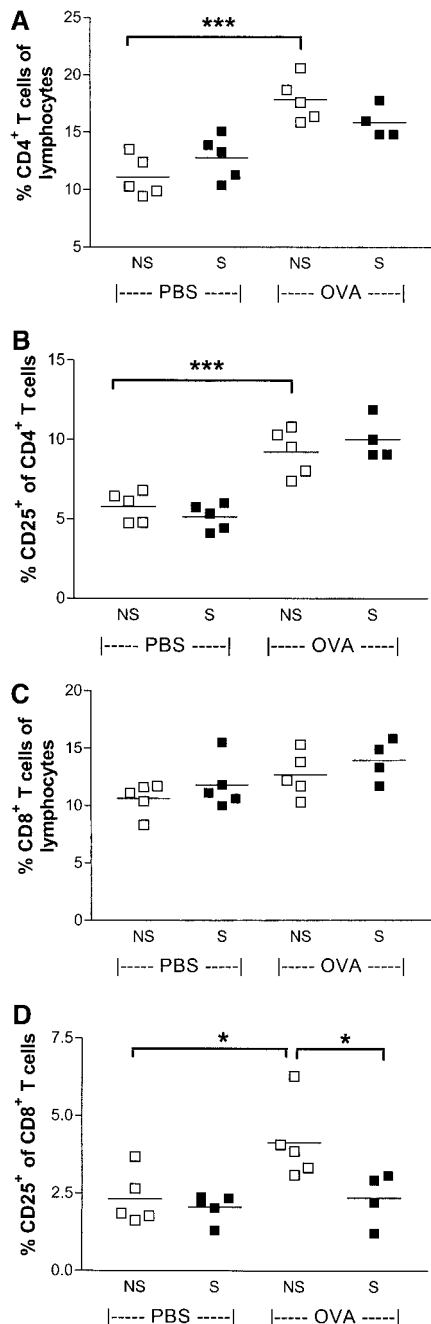


Figure 6. (A and B) CD4⁺ T cells, expressed as percentage of total lymphocytes in lung cell isolates (A) and activated (CD25⁺) CD4⁺ T cells, expressed as percentage of CD4⁺ T cells in lung cell isolates (B) in mice treated with PBS (left) or OVA (right) after being exposed to room air (NS, open squares) or after smoking (S, filled squares). (C and D) CD8⁺ T cells, expressed as percentage of lymphocytes in lung cell isolates (C) and activated (CD25⁺) CD8⁺ T cells, expressed as percentage of CD8⁺ T cells in lung cell isolates (D) in mice treated with PBS (left) or OVA (right) after being exposed to room air (NS, open squares) or after smoking (S, filled squares). **P* < 0.05, ****P* < 0.001.

Previous studies have shown that the induction of allergic airway inflammation and the accompanying AHR were associated with an increase in eosinophils (13), macrophages (14, 15), and CD4⁺ T cells in lung tissue (13, 16–18), an increase in Th2 cytokines in BAL fluid (19), and an increase in OVA-specific IgE in serum (20, 21). Our model met most of these characteris-

TABLE 3. Number of macrophages and neutrophils in lung tissue sections (*n* = 3, mean ± SEM)

Cell type	PBS-NS	PBS-S	OVA-NS	OVA-S
Macrophages, no./mm ²	432 ± 16	566 ± 10*	710 ± 33 [†]	541 ± 20 [‡]
Neutrophils, no./mm ²	108 ± 28	275 ± 17 [†]	151 ± 7	122 ± 9

**P* < 0.05 PBS-S versus PBS-NS.

[†] *P* < 0.001 PBS-S versus PBS-NS.

[‡] *P* < 0.001 PBS-NS versus OVA-NS.

[§] *P* < 0.01 OVA-S versus OVA-NS.

tics. To our surprise, however, our study showed no apparent negative effects of *short-term* smoking on these parameters in mice with OVA-induced airway inflammation.

Three weeks of smoking significantly ameliorated both OVA-induced bronchoconstriction and responsiveness to MCh compared with nonsmoking asthmatic mice. These results corresponded with a significant reduction of eosinophils in BAL fluid and a similar pattern in lung tissue of smoking OVA-mice compared with their nonsmoking controls. A reduced number of eosinophils may explain the observed improved responses on OVA and MCh provocation, because mediators derived from eosinophils are known to induce bronchoconstriction and AHR (22–24). Similar to asthmatic mice that smoke, a reduction of eosinophil numbers has been found in human smokers with asthma (4). However, this was observed in a cross-sectional study, and the results may have been biased by the fact that patients with asthma who could not tolerate smoking were not studied. It is well conceivable that, in particular, individuals with asthma that tolerate smoking may have less severe symptoms and thus likely less severe airway inflammation.

In view of the well-known detrimental effects of long-term smoking (3, 10–12), the observed reduction in allergic airway inflammation in mice after 3 wk of smoking is very puzzling. The acute effects of cigarette smoke caused a change in the pulmonary inflammation process, but further studies have to show whether this is a temporary change. One of the changes in the inflammation process was an altered response of macrophages. The induction of allergic airway inflammation alone induced a significant increase of macrophages in the lung in our protocol, as demonstrated before (14). The same was found for smoking alone in our protocol and in that of others (25). Surprisingly, in lungs of asthmatic animals smoking reduced (but not normalized) the elevated number of macrophages.

Smoking also altered T cell responses in our model. The influx of CD4⁺ T cells was inhibited by smoking, though this difference did not reach statistical significance. Considering the important role of CD4⁺ T cells in allergen-induced eosinophilic inflammation and AHR (13, 17, 18), it may be possible that smoking caused a change in the balance of the Th1 and Th2 cells within the lung, thereby dampening the allergic airway inflammation. Changes in the specific Th1 and Th2 cytokines could have given us more insight into this hypothesis. Unfortunately, the cytokine levels in BAL fluid were too low for meaningful interpretation.

Another possible explanation for the apparent (short-term) dampening effects of smoking on allergic airway inflammation is the induction of “protective” enzymes like inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1). These enzymes produce nitric oxide (NO) and carbon monoxide (CO), respectively, both shown to inhibit allergic airway inflammation (26–28). Both enzymes are upregulated after smoking (29, 30) and either one alone or the combination may be responsible for

the inhibitory effects of short-term smoking on inflammation. We have examined the frozen tissue sections of lung tissue of all our experimental groups ($n = 3$) for the expression of iNOS and found an upregulation of expression in nonsmoking OVA-exposed animals as compared with the nonsmoking PBS-exposed animals (data not shown). In the PBS-exposed animals, smoking induced an upregulation of expression of iNOS, as was found before by Chang and coworkers (29). In the OVA-exposed animals, however, smoking had no effect on the iNOS expression. An anti-inflammatory role for iNOS is therefore not likely in this case. Further studies will have to elucidate the role of HO-1 and the CO it produces. There may, however, also be a role for exogenously produced CO. Our smoking mice have been exposed to high levels of CO (HbCO levels directly after smoking of $22.5 \pm 1.7\%$; B.W.A. van der Strate, unpublished observations). Recent work from Choi and coworkers showed that OVA-sensitized and -challenged mice exposed to comparable levels of CO exhibited reduced MCh responsiveness (31) and reduction of eosinophils in BAL fluid compared with control OVA-mice exposed to air (26). Our similar results could therefore also be explained in part by effects of CO generated from a burning cigarette. This hypothesis is currently under investigation.

In conclusion, we have demonstrated for the first time and to our surprise that smoking for 3 wk in mice with established allergic airway inflammation ameliorates asthma features in this mouse model, i.e., the response to OVA, the severity of AHR, and the accompanying airway inflammation. These data in mice contrast with the effects of long-term cigarette smoking on lung function of humans with asthma (3) and are likely explained by the short time period of smoke exposure in mice, perhaps in combination with a species difference. During this short time the direct effects that may be caused by, for instance, CO can prevail above effects that need more time to cause changes like remodeling. Further experiments will therefore have to focus on the effects of long-term smoking in this asthma mouse model.

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