

Short-Term Cigarette Smoke Exposure Enhances Allergic Airway Inflammation in Mice

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Rationale: Epidemiologic studies suggest that tobacco smoke contributes to the prevalence and occurrence of exacerbations in asthma. The effect of active smoking in adolescents with atopy is poorly understood. **Objectives:** We developed an experimental model to investigate the influence of smoking on antigen-induced airway inflammation and airway responsiveness in mice that were previously sensitized. **Methods:** Ovalbumin (OVA)-sensitized BALB/c mice were exposed to air or mainstream smoke (5 days/week) and to phosphate-buffered saline (PBS) or OVA aerosol (3 times/week) for 2 weeks ($n = 8$ for each group). **Results:** Airway responsiveness to intravenously injected carbachol was increased ($p < 0.05$) in smoke- and OVA-exposed mice compared with all other groups. There was an additive effect of smoke and OVA exposure on total cell numbers, macrophages, and dendritic cells in bronchoalveolar lavage fluid and on CD4⁺ and CD8⁺ T lymphocytes and dendritic cells in lung tissue ($p < 0.05$ compared with mice exposed to smoke and PBS and to mice exposed to air and OVA). Concurrent smoke and OVA exposure augmented OVA-specific IgE in serum compared with air and OVA exposure. In lavage fluid supernatant, eotaxin was increased in air- and OVA-exposed mice. The further increase observed in the group exposed to both OVA and cigarette smoke came close to formal significance ($p = 0.06$). Thymus- and activation-regulated chemokine was augmented in mice exposed to either smoke or OVA, without additional effect. **Conclusions:** Our data indicate that acute concurrent exposure to allergen and mainstream cigarette smoke enhances airway inflammation and airway responsiveness in previously sensitized mice.

Keywords: additive; asthma; cytokines; hyperresponsiveness; severity

Epidemiologic studies indicate that airway pollutants, such as tobacco smoke, contribute to the development and the increase in the severity of asthma. Active as well as passive smoking is positively associated with asthma severity (1–9). Exposure to cigarette smoke results in more frequent asthma attacks, more asthma symptoms (1), a lower lung function (2), an accelerated decline in lung function (2, 3), and higher asthma severity scores (4). Moreover, active cigarette smoking impairs the efficacy of short-term inhaled corticosteroid treatment in steroid-naïve patients with asthma (5) and the efficacy of oral corticosteroids in patients with chronic stable asthma (6). The severity of asthma is strongly correlated with current smoking (7), and some studies suggest that there is an increased risk for bronchial asthma in persons who have smoked for 3 years or more (8). Adolescents

who start smoking might thus be at increased risk for the development of asthma. Little is known about the effect of active smoking in adolescents already sensitized to an allergen. In some epidemiologic studies that assessed the effect of the interaction between smoking and atopy on asthmalike symptoms (9–11), atopy and smoking were found to be independent risk factors for the development of asthma during adolescence. To understand the inflammatory mechanisms involved in young adults with atopy who start smoking, we developed an experimental model. In this model, the influence of smoking on airway inflammation and airway responsiveness in BALB/c mice that were previously sensitized was investigated.

Some of the results of these studies have been previously reported in the form of an abstract (12).

METHODS

Animals

Male inbred BALB/c mice of about 8 weeks old were obtained from Harlan CBP (Zeist, the Netherlands).

Immunization

All mice were immunized by an intraperitoneal injection of 10 μ g ovalbumin (OVA) adsorbed to 1 mg aluminum hydroxide on Day 0 and boosted on Day 7.

In Vivo Tobacco Smoke Exposure

Mainstream cigarette smoke exposures were performed four times a day, 5 days a week, with five Kentucky Reference cigarettes (1R3) per five mice per exposure. Details are provided in the online supplement.

OVA Exposure and Experimental Protocol

Air or smoke exposures took place 5 days a week from Day 14 until Day 24. At Days 14, 16, 18, 21, and 24 (30 minutes after air or smoke exposure) mice were exposed to OVA (1%) (details provided online) or phosphate-buffered saline (PBS) aerosol during 30 minutes ($n = 8$ in each group).

Assessment of Airway Responsiveness

Airway responsiveness to carbachol was measured 24 hours after the final allergen exposure. More details are provided in the online supplement. Lung resistance, induced by increasing doses of carbachol, was evaluated with a computerized pulmonary mechanics analyzer (Mumed lung function recording system, version 5.0; Mumed Systems Ltd., London, UK). The concentration of carbachol causing a 50% increase of baseline resistance was calculated.

Bronchoalveolar Lavage and Lung Digest

Immediately after the assessment of airway responsiveness, bronchoalveolar lavage (BAL) was performed via intratracheal instillation of 1 ml Hank's balanced salt solution (Pasteur, Brussels, Belgium) plus 1% bovine serum albumin for cytokine measurements. Three instillations with 1 ml of Hank's balanced salt solution were performed to collect cells for total and differential (cytospin analysis) cell counts. The remaining cells and cells from lung digest were analyzed by fluorescence activated cell sorter. (See online supplement for details.)

Measurement of Total and OVA-specific IgE

Blood was drawn from the heart for measurement of total and OVA-specific IgE with ELISA. (See online supplement for details.)

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Immunofluorescent Labeling and Flow Cytometry

Flow cytometry data were acquired on a FACSVantage SE flow cytometer running CELLQuest 3.0 software (Becton Dickinson, San Jose, CA). FlowJo software (Treestar, San Carlos, CA) was used for data analysis. (See online supplement for details.)

Cytokine and Chemokine Measurements

Measurements have been performed on supernatant of lavage fluid. Interleukin 5 (IL-5) was measured with a sensitive bioassay with a detection limit at approximately 5 pg/ml, as previously described (13). Eotaxin (sensitivity, 3 pg/ml), IFN- γ (sensitivity, 2 pg/ml), thymus- and activation-regulated chemokine (TARC; sensitivity, < 5 pg/ml), tumor necrosis factor α (sensitivity, < 5.1 pg/ml), and IL-13 (sensitivity, 1.5 pg/ml) were measured with ELISA (R&D Systems Europe, Abingdon, UK).

Statistical Analysis

Reported values were expressed as mean $\pm$ SEM, and *p* values of less than 0.05 were regarded as significant. For the statistical analysis, the SPSS program was used (SPSS 11.0; SPSS, Inc., Chicago, IL). All outcome variables were compared using nonparametric tests (Kruskal-Wallis, Mann-Whitney U test with Bonferroni's corrections). (See online supplement for details.)

For measurements of bronchial responsiveness, cumulative dose-response curves for the changes in lung resistance with increasing doses of carbachol were constructed. The changes in lung resistance were expressed as percentage increase in lung resistance. The cumulative dose-response curves were compared through analysis of variance with *post hoc* (least significant difference and Scheffé) tests.

RESULTS

BAL Fluid

BAL fluid (BALF) of immunized mice exposed to cigarette smoke or aerosolized OVA contained an increased amount of cells compared with air- and PBS-exposed immunized animals as indicated in Table 1. Combined exposure to cigarette smoke and OVA aerosol had an additive effect on total cell number in BALF.

OVA exposure increased the number of eosinophils in BALF, whereas cigarette smoke exposure increased the number of neutrophils in BALF (Table 1).

Smoke or OVA exposure as such did not increase the number of macrophages in BALF. Cigarette smoke synergized with OVA to significantly elevate the total number of macrophages in BALF ($p < 0.05$; Table 1). The lymphocyte number in BALF was increased from exposure to tobacco smoke or to OVA

($p < 0.001$), but was not further increased by combination of both stimuli (Table 1).

The number of dendritic cells (DCs) in BALF was increased after exposure to either cigarette smoke or OVA. Exposure to both stimuli further increased the amount of DCs recovered from BALF (Table 1).

Measurement of Total and OVA-specific IgE

Total IgE in serum was not increased because of exposure to cigarette smoke as such. In mice challenged with OVA, a significant increase in total IgE was observed (Table 2).

OVA-specific IgE was significantly higher in mice exposed to OVA compared with PBS-exposed mice. The further increase observed in the group that was simultaneously exposed to OVA and cigarette smoke came close to formal significance ($p = 0.06$; Table 2).

Lung Tissue: Fluorescence-activated Cell Sorter Analysis

There were no significant differences in total cell numbers in lung tissue between the four groups (data not shown), although some shifts were seen in cell lineages: DCs and activated CD4⁺ and CD8⁺ T lymphocytes (Figure 1) were increased by exposure to either cigarette smoke or OVA. Simultaneous smoke and OVA exposure had an additive effect on the three cell types.

Airway Responsiveness

Figure 2 shows the percentage of increase in lung resistance with increasing doses of carbachol. After 2 weeks of cigarette smoke or OVA exposure, the dose-response curve for carbachol was not significantly different from those of control animals. In the group exposed to both OVA and cigarette smoke, airway responsiveness significantly increased compared with all other groups.

Cytokine and Chemokine Measurements in BALF Supernatant

IL-5, IFN- γ , and tumor necrosis factor α were not detectable in the supernatant of BALF. Eotaxin was significantly increased ($p < 0.05$) in the group exposed to OVA. The further increase observed in the group that was simultaneously exposed to OVA and cigarette smoke came close to formal significance ($p = 0.06$; Table 2). TARC was augmented significantly ($p < 0.001$) in the group exposed to either cigarette smoke or OVA. The group exposed to both stimuli showed TARC levels that were similar to the group only exposed to smoke (Table 2).

Exposure to OVA increased IL-13 in BALF supernatant ($p < 0.001$); concurrent cigarette smoke exposure did not further increase IL-13 (Table 2).

TABLE 1. FINDINGS ON BRONCHOALVEOLAR LAVAGE FLUID

	PBS/NSM	PBS/SM	OVA/NSM	OVA/SM
Total cell number, $\times 10^3$	350.00 $\pm$ 45.15	640.00 $\pm$ 68.03*	1,036.25 $\pm$ 237.45 [‡]	2,317.50 $\pm$ 330.89 [§]
Neutrophils, $\times 10^3$	0.22 $\pm$ 0.14	198.93 $\pm$ 29.07 [†]	16.79 $\pm$ 8.32 [§]	180.67 $\pm$ 27.07**
Macrophages, $\times 10^3$	348.69 $\pm$ 45.03	420.98 $\pm$ 46.33	371.29 $\pm$ 69.49	1,096.47 $\pm$ 221.15 [§]
Lymphocytes, $\times 10^3$	1.01 $\pm$ 0.68	9.00 $\pm$ 1.68 [†]	37.33 $\pm$ 9.53 [§]	35.43 $\pm$ 8.54
Eosinophils, $\times 10^3$	74.75 $\pm$ 74.75	11.08 $\pm$ 4.80	610.87 $\pm$ 170.97 [§]	1,004.94 $\pm$ 200.33 [§]
Dendritic cells, $\times 10^3$	3.00 $\pm$ 0.50	12.08 $\pm$ 1.85*	17.79 $\pm$ 4.08	31.24 $\pm$ 7.59

Definition of abbreviations: NSM = sham-exposed; OVA = ovalbumin; PBS = phosphate-buffered saline; SM = smoke-exposed. Values are reported as mean $\pm$ SEM; *n* = 8/group.

* $p < 0.01$.

[†] $p < 0.001$ versus sham-exposed, similarly PBS-exposed animals.

[‡] $p = 0.06$.

^{||} $p < 0.01$.

[§] $p < 0.001$ compared with PBS-exposed, similarly sham- or smoke-exposed animals.

[¶] $p < 0.01$.

** $p < 0.001$ versus sham-exposed, similarly OVA-exposed animals.

TABLE 2. EOTAXIN, THYMUS- AND ACTIVATION-REGULATED CHEMOKINE, INTERLEUKIN 13 IN BRONCHOALVEOLAR LAVAGE FLUID SUPERNATANT, TOTAL IgE, OVALBUMIN-SPECIFIC IgE IN SERUM

	PBS/NSM	PBS/SM	OVA/NSM	OVA/SM
Total IgE, $\mu\text{g/ml}$	0.35 ± 0.05	0.40 ± 0.09	$2.59 \pm 0.67^{\dagger}$	$2.33 \pm 0.48^{\S}$
OVA-specific IgE, U/ml	6.10 ± 0.23	6.50 ± 0.94	$55.37 \pm 8.1^{\ddagger}$	$86.18 \pm 12.90^{\parallel}$
Eotaxin, pg/ml	1.38 ± 0.53	0.61 ± 0.54	$11.32 \pm 3.12^{\ddagger}$	$26.98 \pm 6.36^{\parallel}$
TARC, pg/ml	0.17 ± 0.12	$41.75 \pm 3.77^*$	$303.63 \pm 89.27^{\S}$	$40.77 \pm 5.02^{\ddagger}$
IL-13, pg/ml	0.0 ± 0.0	0.0 ± 0.0	$27.51 \pm 6.58^{\S}$	$31.98 \pm 8.10^{\ddagger}$

Definition of abbreviations: IL-13 = interleukin 13; NSM = sham-exposed; OVA = ovalbumin; PBS = phosphate-buffered saline; SM = smoke-exposed; TARC = thymus- and activation-regulated chemokine.

Values are reported as mean $\pm$ SEM; n = 8/group.

* p < 0.001 versus air-exposed, similarly PBS-exposed animals.

† p < 0.01.

‡ p < 0.05.

§ p < 0.001 compared with PBS-exposed, similarly air- or smoke-exposed animals.

$^{\parallel}$ p = 0.06.

‡ p < 0.001 versus air-exposed, similarly OVA-exposed animals.

DISCUSSION

This study examined the effect of concurrent exposure to allergen and cigarette smoke in a murine model of allergic airway inflammation. As expected, immunized BALB/c mice challenged with OVA developed an eosinophilic airway inflammation, which was associated with increased numbers of lymphocytes and DCs in lavage and lung tissue. Exposure of mice to cigarette smoke induced a neutrophilic influx. Sensitized mice exposed to both OVA and cigarette smoke were found to have a pulmonary inflammation with characteristics of both smoke- and allergen-induced inflammation. OVA-specific IgE in serum as well as Th2-cytokine levels in BALF supernatant were increased when OVA and cigarette smoke exposure were combined. These changes were associated with increased airway responsiveness.

It is well documented that cigarette smoking causes an accumulation of neutrophils and macrophages in human lung tissue (14–16) and BALF (17). Neutrophils in BALF were also increased in our model with exposure of mice to mainstream smoke. In a study by Chalmers and others (18), total sputum cell counts were higher in smokers with asthma than in healthy nonsmokers, healthy smokers, and nonsmokers with asthma. The authors suggested that the neutrophilic inflammation related to smoking may be additive to the underlying asthmatic airway inflammation. Seymour and colleagues (19) used a murine model of allergy to demonstrate that environmental tobacco smoke could amplify an ongoing allergic response (19). In that study, sidestream cigarette smoke was aged and diluted in conditioning

chambers for 2 minutes, and then further diluted with fresh air, whereas in our model, mainstream cigarette smoke was used. In a recent report, Melgert and coworkers (20) described an attenuation of OVA-induced airway inflammation when allergic mice were exposed to cigarette smoke. At first sight, the results obtained in this study are contradictory to the results we found. However, these divergent results can be explained by the different experimental design, and thus, in fact, both studies are complementary rather than conflicting. Our study is a model of acute simultaneous exposure of allergen and cigarette smoke over a period of days, whereas Melgert and coworkers (20) used a more chronic model of airway inflammation, in which C57Bl/6j mice were challenged with antigen aerosol for 4 weeks before the exposure to tobacco smoke started. A nose-only exposure of mice to mainstream smoke was used during 3 weeks, resulting in carboxyhemoglobin (HbCO) levels of approximately 22% directly after smoking. It is known that exogenously administered CO attenuates airway inflammation (21) and hyperresponsiveness (22) in mice, and this can explain the suppressive effects Melgert and coworkers (20) observed. In our model, HbCO immediately after smoke exposure was $8.29 \pm 1.4\%$ (n = 7), according well with previous reports (23) and with percentage of HbCO in peripheral blood of human smokers (4–10%) (24).

In our model, DCs in BALF and in lung tissue were increased in mice that were exposed to either OVA or cigarette smoke. The further increase in DCs when smoke and OVA were combined

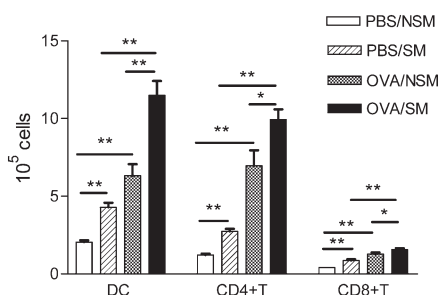


Figure 1. Dendritic cells (DCs), activated CD4⁺ and CD8⁺ T lymphocytes in lung tissue (n = 8/group). NSM = air-exposed; OVA = ovalbumin; PBS = phosphate-buffered saline; SM = smoke-exposed. Values are reported as mean $\pm$ SEM. *p < 0.05, **p < 0.001.

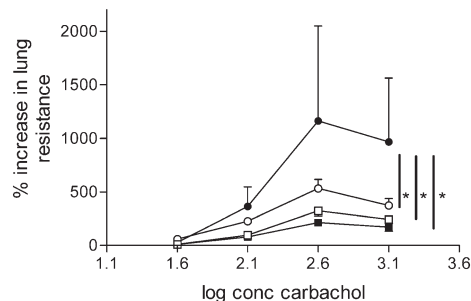


Figure 2. Airway responsiveness to carbachol in sensitized BALB/c mice exposed to PBS and air (open squares), PBS and smoke (open circles), OVA and air (closed squares), and OVA and smoke (closed circles) for 2 weeks (n = 8/group). Values are reported as mean $\pm$ SEM. *p < 0.05. Conc = concentration.

suggests that the increase in DCs related to smoking may be additive to the underlying allergic airway inflammation. DCs can secrete chemotactic factors that attract other inflammatory cells, such as neutrophils, macrophages, natural killer cells, and more DCs, and they are also important in the induction and maintenance of eosinophilic airway inflammation (25). Activated CD4⁺ and CD8⁺ T cells were increased in mice exposed to tobacco smoke, with a predominance of the number of CD4⁺ T cells. A similar observation was made in guinea pigs (26). In humans, however, the inflammatory response to tobacco smoke was mostly characterized by increases of CD8⁺ T lymphocytes rather than CD4⁺ T cells (27). Activated CD4⁺ and CD8⁺ T cells were increased in lungs of mice that were aerosolized with OVA. In this study, activated CD4⁺ T lymphocytes were increased when mice were exposed to both smoke and OVA versus mice exposed to a single stimulus. Other investigators previously showed that an accumulation of activated CD4⁺ T lymphocytes was associated with the development of airway hyperresponsiveness (28, 29). It may come as a surprise that OVA exposure as such did not induce hyperresponsiveness, but under the current circumstances, there was no need to obtain a shift in the dose-response curve by exposure to OVA only. On the contrary, our experiments were set up to examine the interaction between OVA and cigarette smoke, and our results indeed show an increase in airway responsiveness when mice are exposed to both OVA and cigarette smoke.

The higher nonspecific airway responsiveness observed in mice exposed to both OVA and smoke could be a functional consequence of the large increase in activated CD4⁺ T lymphocytes, secreting a whole range of cytokines. Also, the increased number of eosinophils may help explain this phenomenon, because mediators derived from eosinophils are believed to induce airway hyperresponsiveness (21, 30). Another cell type that plays a role in the development of airway hyperresponsiveness is CD8⁺ T lymphocytes (31, 32). In our model, an additive effect was seen on CD8⁺ T lymphocytes when smoke and OVA exposure were combined, further explaining the observed lung function data. To further unravel the mechanisms behind our observations, cytokine measurements have been performed on BALF supernatant. When sensitized mice are exposed to allergen, a T-helper 2 (Th2) response was seen. In the present experiment, an increase was seen in TARC, IL-13, and eotaxin in BALF supernatant when mice were sensitized and challenged with OVA. These findings clearly indicate that our model is Th2-driven. TARC was elevated in murine lungs during the allergic inflammation after OVA exposure, as previously described by Kawasaki and coworkers (33). Their results indicated that TARC is a pivotal chemokine for the induction of CD4⁺ Th2 lymphocyte and eosinophil infiltration in the airways. In our mice, TARC was also elevated significantly in OVA-exposed mice, but to our surprise, a concurrent exposure to cigarette smoke appeared to have an inhibiting effect. Therefore, in our model, the massive increase in activated CD4⁺ T cells in both OVA- and smoke-exposed mice can only be partly explained by an increase in TARC levels, so it is likely that other C-C chemokines, such as eotaxin or RANTES (regulated on activation, normal T-cell expressed and secreted)/CCL5, play a more important role in this process (34).

IL-13 was increased in mice exposed to OVA versus PBS-exposed mice. Tobacco smoke exposure did not amplify this response. IL-13 regulates IgE production, eosinophil recruitment, and airway hyperresponsiveness (35), and it is the most potent inducer of eotaxin (36), an eosinophil-selective C-C chemokine produced primarily by respiratory epithelial cells. Eotaxin in BAL supernatant was increased in our OVA-exposed mice, whereas a concurrent exposure to smoke had an adjuvant

effect. The increased eotaxin level in BAL supernatant was associated with a marked infiltration of the airways with eosinophils. MacLean and colleagues (37) suggested that eotaxin is one of the molecular links between antigen-specific T-cell activation and the recruitment of eosinophils into the airways. Because eotaxin has been demonstrated to partially regulate eosinophil recruitment during the late-phase response (38), it might be possible that a higher amount of eosinophils still had to arrive into the airways at the moment we killed the mice. Furthermore, it may be possible that the influx of other inflammatory cells into the airways because of smoke exposure causes changes in eosinophil recruitment. Our data indicate that acute concurrent exposure to allergen and mainstream cigarette smoke enhances airway inflammation and airway responsiveness in previously sensitized BALB/c mice. These results support the hypothesis that the development of asthmatic symptoms in young adults with atopy is enhanced by starting active smoking.

Conflict of Interest Statement: K.B.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; R.A.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; G.F.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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