



Cigarette smoke dissociates inflammation and lung remodeling in OVA-sensitized and challenged mice[☆]

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ABSTRACT

We evaluated the effects of cigarette smoke (CS) on lung inflammation and remodeling in a model of ovalbumin (OVA)-sensitized and OVA-challenged mice. Male BALB/c mice were divided into 4 groups: non-sensitized and air-exposed (control); non-sensitized and exposed to cigarette smoke (CS), sensitized and air-exposed (OVA) (50 µg + OVA 1% 3 times/week for 3 weeks) and sensitized and cigarette smoke exposed mice (OVA + CS). IgE levels were not affected by CS exposure. The increases in total bronchoalveolar fluid cells in the OVA group were attenuated by co-exposure to CS, as were the changes in IL-4, IL-5, and eotaxin levels as well as tissue elastance ($p < 0.05$). In contrast, only the OVA + CS group showed a significant increase in the protein expression of IFN- γ , VEGF, GM-CSF and collagen fiber content ($p < 0.05$). In our study, exposure to cigarette smoke in OVA-challenged mice resulted in an attenuation of pulmonary inflammation but led to an increase in pulmonary remodeling and resulted in the dissociation of airway inflammation from lung remodeling.

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1. Introduction

Epidemiologic studies have shown that tobacco smoke contributes to the development and increased severity of asthma (Melgert et al., 2004; Moerloose et al., 2005). Cigarette smoke exposure results in more frequent asthma attacks and symptoms, impairment in lung function and decreased efficacy of short-term inhaled corticosteroid treatment in steroid-naïve patients with asthma (Althius et al., 1999; James et al., 2004; Siroux et al., 2000). Although some clinical trials suggest that smokers have a lower risk of developing asthma symptoms when compared with nonsmokers and ex-smokers (Hjern et al., 2001; McWhorter et al., 1989; Tsoumakidou et al., 2007), such findings should be interpreted

carefully due to the behavior of some aspects of the asthmatic inflammatory process (Churg et al., 2006; Trimble et al., 2009). Studies with animal models involving cigarette smoke and allergic asthma have shown conflicting results, especially regarding lung inflammation and remodeling (Melgert et al., 2004; Min et al., 2007; Moerloose et al., 2005; Robbins et al., 2005). Some studies have shown that short-term exposure to environmental tobacco smoke in experimental models of asthma in mice induces augmented levels of airway remodeling associated with an increase of eosinophils in bronchoalveolar lavage fluid (Min et al., 2007; Moerloose et al., 2005). However, others have demonstrated a decrease of inflammatory cells after short-term smoke exposure in allergic mice (Melgert et al., 2004; Robbins et al., 2005).

Airway inflammation and lung remodeling are distinguishing features observed in both clinical and experimental asthma, as well as in cigarette smoke exposure, and these features are clearly related to airflow obstruction (Churg et al., 2006). The pathogenesis of airway inflammation and lung remodeling in lung disorders involving cigarette exposure is a field fraught with disagreement (Jeffery, 2001). There are two main schools of thought on the subject: one holds that lung remodeling is a response to repeated inflammatory injuries caused by cigarette smoke exposure and

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represents a trend toward developing abnormal inflammatory reactions to small stimuli (Jeffery, 2001). This point of view accounts for changes in airway structure as an exaggerated healing process by inflammatory cells. Another perspective is that lung remodeling is a product of the excessive release of growth factors (e.g., TGF- β and collagen types I and III), leading to an incremental increase in fibrotic tissue and muscle thickness. These growth factors could be a direct response to the provocative agents mediated by chronic injury or repair of airway epithelium but not directly dictated by the inflammatory response (Chapman, 2004; Churg et al., 2006; Gauldie et al., 2002; Kenyon et al., 2003; Selman et al., 2001). These findings suggest that inflammation and fibrosis may occur independently (Chapman, 2004; Gauldie et al., 2002; Selman et al., 2001).

Therefore, we reasoned that cigarette smoke exposure could cause opposite effects on airway inflammation, responsiveness and pulmonary remodeling in asthma. In the present study, we used an experimental model of allergic inflammation in BALB/c mice to investigate the effects of three weeks of mild cigarette smoke exposure on pulmonary inflammation and lung remodeling when both stimuli (i.e., allergen challenge and cigarette smoke) are administered simultaneously.

2. Methods

2.1. Animals and experimental design

Thirty-one male BALB/c mice (20–25 g) from the vivarium of the School of Medicine, University of Sao Paulo were divided into 4 groups as follows: animals non-sensitized and air-exposed (control group, $n=8$); animals non-sensitized and exposed to cigarette smoke (CS group, $n=7$); animals sensitized and air-exposed (OVA group, $n=7$); and animals sensitized and exposed to cigarette smoke (OVA+CS group, $n=9$). This study was approved by the Review Board for Human and Animal Studies of the School of Medicine of the University of Sao Paulo. All animal care and experimental procedures followed the EU Directive, 2010/63/EU for animal experiments guidelines (Official Journal of the European Union, 2010).

2.2. OVA model

We used a modified OVA protocol from Vieira et al. (2007). BALB/c mice were sensitized by intraperitoneal (i.p.) injection of aluminum hydroxide-adsorbed ovalbumin (OVA) (50 μ g per mouse) or saline (NaCl 0.9%) on days 0, 14 and 28. Twenty-one days after the first i.p. injection, mice were exposed to aerosolized OVA (1%) or saline 3 times per week for 30 min in the morning until day 42 (Fig. 1A).

2.3. Cigarette smoke exposure

We used a modified cigarette smoke exposure protocol from Biselli et al. (2011) beginning 21 days after the first immunization and lasting until day 42, as in the OVA protocol. CS and OVA+CS mice were exposed to cigarette smoke for 30 min per day and 5 days per week for 3 weeks in the afternoon (Fig. 1A and B). The flow rate was set at 1.5 L/min, which produced carbon monoxide (CO) levels ranging from 300 to 350 ppm and resulted in blood levels of carboxyhemoglobin of 10%.

2.4. Respiratory mechanics

Forty-eight hours after the last challenge, the animals were anesthetized with pentobarbital sodium (50 mg/kg i.p.), and a tracheotomy was performed. The mice were then connected to a

ventilator for small animals (flexiVent, Scireq, Quebec, Canada) with the tidal volume and frequency set at 20 mL/kg and 2 Hz, respectively. After 1 min, the animals were paralyzed with pancuronium bromide (1 mg/kg), and the anesthetic level was checked during the entire procedure. Oscillatory lung mechanic measurements were performed to obtain airway resistance (Raw), small airway resistance (Gtis) and tissue elastance (Htis) (Hantos et al., 1992).

2.4.1. Methacholine dose–response curve

Different methacholine concentrations, ranging from 6 to 50 mg/mL, were delivered by an ultrasonic device over 1 min (Respira Max, NS, LTDA, Sao Paulo, Brazil). After 30 s, respiratory mechanics data were collected. A response curve for bronchial responsiveness was performed immediately after the methacholine challenge, and bronchoalveolar fluid (BALF) and blood were collected. The animals were euthanized by rapid exsanguination via the abdominal aorta while anesthetized.

2.5. Measurement of serum IgE

Total serum IgE was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Pharmingen, San Diego, CA) following the manufacturer's protocol.

2.6. Total and differential cell counting in bronchoalveolar lavage fluid (BALF)

The lungs were gently lavaged with 3 instillations of 0.5 mL of phosphate buffered saline (PBS, pH 7.2) via tracheal cannula. Total cells were counted in a Neubauer's hemocytometer chamber. Differential cell counts of 300 cells/animal were obtained after Diff Quick staining of BALF prepared on slides. All measurements were taken in a blinded fashion.

2.7. Bio-Plex assay for IL-4, IL-5, eotaxin, IL-10, IFN- γ , GM-CSF and VEGF from lung tissue

A mouse 7-Plex cytokine assay kit (Millipore Laboratories, Inc., Merck KGaA, Darmstadt, Germany) was used to test samples for the presence of 7 cytokines. The assay was read on the Bio-Plex suspension array system. The data were normalized to the amount of input tissue.

2.8. Lung histology and morphometry

The right lungs were fixed in formalin and embedded in paraffin. Five-micrometer-thick sections were stained with picosirius red (PS) for collagen fibers. Immunohistochemistry (IHC) was performed with anti-IL4, anti-IL-5, anti-IL-10 and anti-TGF- β antibodies (Santa Cruz, CA), as previously described (de Magalhães Simoes et al., 2005). Measurements of collagen content and IHC-positive cells were performed with imaging analysis software (Image-Pro Plus, 4.5.0.29 for Windows, Media Cybernetics, Silver Spring, MD) on images acquired from a light microscope with a digital camera connected to a computer (Leica DMR; Leica Microsystems, Wetzlar GmbH, Wetzlar, Germany). Analyses were made from five images of a transversally cut airway and its adjacent vascular structure. The collagen content was measured within the adventitial space between the airway and the adjacent blood vessel (peribronchovascular space) as the collagen area divided by the respective basement membrane length ($\mu\text{m}^2/\mu\text{m} = \mu\text{m}$). IL-4- and IL-5-positive cells were also measured in the peribronchovascular space, where the infiltration by inflammatory cells in this murine model is more intense (Vieira et al., 2007; Arantes-Costa et al., 2008). TGF- β - and IL-10-positive cells were measured in the

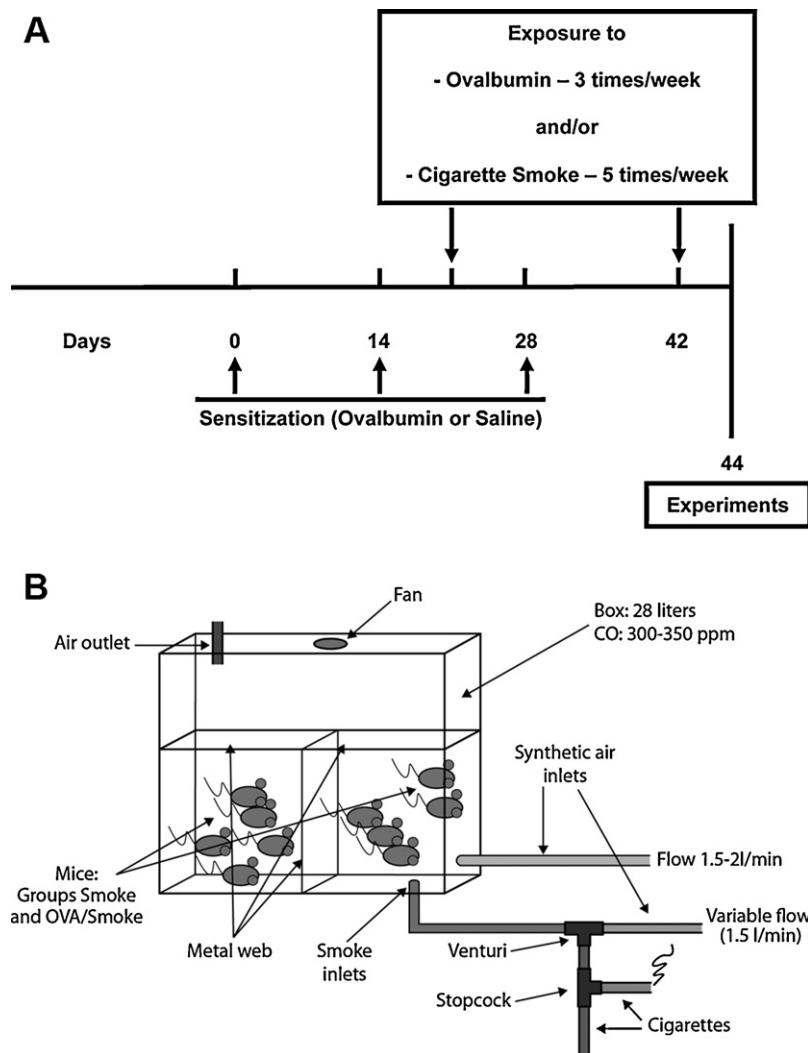


Fig. 1. (A) Experimental design of the study: 3 ovalbumin injections were administrated on days 0, 14 and 28. From days 21 to 42, the animals were exposed to aerosolized OVA or saline (3 days/week for 30 min) in the morning and to cigarette smoke (5 days/week for 30 min) in the afternoon; 48 h later, anesthetized mice were euthanized. (B) Cigarette smoke exposure device (modified from Biselli et al., 2011): cigarette smoke exposure was performed in a 28-L plastic box with two inlets (for air and smoke), one outlet and a fan (for the enhancing air and smoke mixture inside the box). In the first inlet, we applied synthetic air (2 L/min). The second inlet received synthetic air that passed through a Venturi valve connected to a lit cigarette that suctioned the cigarette smoke and pumped it inside the box.

bronchial epithelium, in the area between the basement membrane and airway lumen. Cell density was assessed as the number of positive cells divided by the respective basement membrane length (cells/ μm) in 5 bronchovascular structures at a $\times 400$ magnification. All morphometric measurements were performed in a blinded fashion.

2.9. Statistical analyses

Comparisons among groups were performed by a one-way analysis of variance followed by Tukey's post hoc test (parametric data) or by a one-way analysis of variance on ranks followed by Dunn's post hoc test (non-parametric data). The significance level was adjusted to 5% ($p < 0.05$). The correlation between the number of TGF- β -positive cells in the bronchial epithelium and collagen fiber content was performed using Pearson's correlation. For statistical analyses, we used the Sigma Stat 3.5 Software (San Jose, CA).

3. Results

3.1. CS attenuates the eosinophilic inflammation induced by OVA independent of IgE

OVA exposure resulted in a significant increase in lung eosinophils, neutrophils, lymphocytes and macrophages (Table 1). The increases in neutrophils, lymphocytes and macrophages in the BALF were not observed in the group that was exposed to both ovalbumin and cigarette smoke (OVA + CS group). Exposure to cigarette smoke also attenuated the increase in eosinophils induced by OVA exposure; therefore, the numbers of eosinophils observed in the BALF of the OVA + CS group were significantly greater than in the CS group but lower than in the OVA group.

There was an increase in total serum IgE in both of the sensitized mouse groups (OVA and OVA + CS groups) compared with the control and CS groups ($p < 0.001$). Cigarette smoke exposure did not affect this increase in IgE (Fig. 2).

Table 1
Findings of the bronchoalveolar lavage fluid.

	Control	CS	OVA	OVA + CS
Total cells ($\times 10^4/\text{mL}$)	3.07 ± 1.48	2.61 ± 0.49	$19.72 \pm 13.51^*$	5.20 ± 1.63
Eosinophils ($\times 10^4/\text{mL}$)	0.05 ± 0.14	0.01 ± 0.00	$2.73 \pm 0.58^*$	$0.33 \pm 0.08^{**}$
Neutrophils ($\times 10^4/\text{mL}$)	0.07 ± 0.03	0.04 ± 0.05	$0.39 \pm 0.03^*$	0.10 ± 0.13
Macrophages ($\times 10^4/\text{mL}$)	2.23 ± 1.15	1.97 ± 0.25	$8.94 \pm 1.15^*$	3.72 ± 1.66
Lymphocytes ($\times 10^4/\text{mL}$)	0.58 ± 0.31	0.17 ± 0.07	$2.91 \pm 1.57^*$	0.43 ± 0.23

Definition of abbreviations: Control, saline ($n=8$); CS, cigarette smoke; ($n=7$) OVA, ovalbumin ($n=7$); OVA + CS, ovalbumin + cigarette smoke exposure ($n=9$). Values are reported as mean $\pm$ SD.

* $p < 0.005$ versus all Control and OVA + CS experimental groups.

** $p < 0.001$ versus the CS group.

3.2. Decreased tissue elastance after CS exposure in OVA-challenged mice

OVA exposure resulted in higher values of tissue elastance (Htis) compared with the control and CS groups ($p < 0.05$) (Fig. 3A). The values of Htis after methacholine challenge were significantly greater in the OVA group compared with the control group ($p < 0.008$ at concentrations of 6, 12 and 25 mg/mL, and $p < 0.05$ at basal levels and 50 mg/mL). No significant increase in pulmonary elastance response was observed in the OVA + CS group compared with the control and CS groups. There were no significant differences in the Gtis (small airways resistance) or Raw (airways resistance) values among the four experimental groups (Fig. 3B and C).

3.3. Effects of OVA and CS exposure on cytokine expression

IL-4 levels in the lung tissue were increased in the OVA group when compared with all of the other groups ($p < 0.04$) (Fig. 4A). The OVA group also showed a significant increase in IL-4-positive cells in the peribronchovascular compartment ($p = 0.01$ compared with the control group, Fig. 4B). There was no significant difference between the OVA and OVA-CS groups ($p = 0.06$).

The co-exposure to cigarette smoke did not increase IL-5 levels in the lung tissue or the number of IL-5 positive cells in the peribronchovascular space (Fig. 4C and D, respectively). The OVA groups showed a significant increase in IL-5 levels in the lung tissue when compared with all of the other groups ($p = 0.004$); however, this difference could not be detected in the peribronchovascular space, despite graphic similarities ($p = 0.06$).

Cigarette smoke exposure did not increase eotaxin levels in the lung tissue (Fig. 4E). The OVA group showed a significant increase in eotaxin when compared with all of the other groups ($p = 0.01$). In

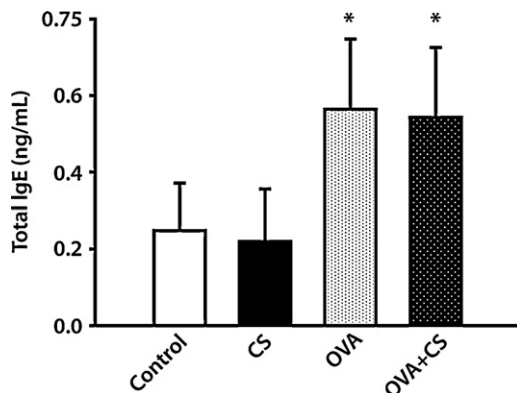


Fig. 2. Serum IgE levels (means and SD). CS, mice exposed to cigarette smoke; OVA, mice exposed to ovalbumin; and OVA + CS, mice exposed to both ovalbumin and cigarette smoke. The values are presented as the means $\pm$ SD. * $p < 0.001$ when compared with the Control and CS groups.

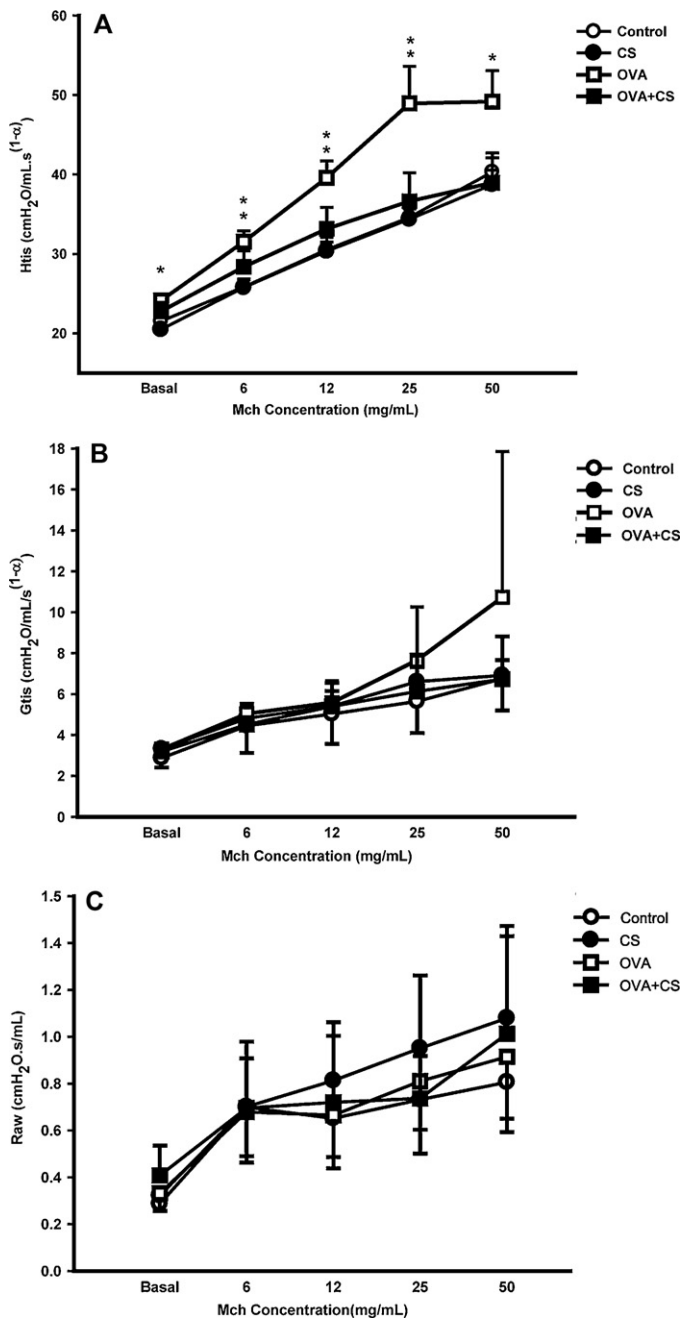


Fig. 3. Dose-response curves to inhaled methacholine solution (Mch). The responses to Mch are measured as Htis (tissue elastance), Gtis (tissue resistance) and Raw (airway resistance) (A, B and C, respectively). The values are presented as the means $\pm$ SD. * $p < 0.05$ when compared with the control and CS groups, ** $p < 0.008$ compared with the control and CS groups.

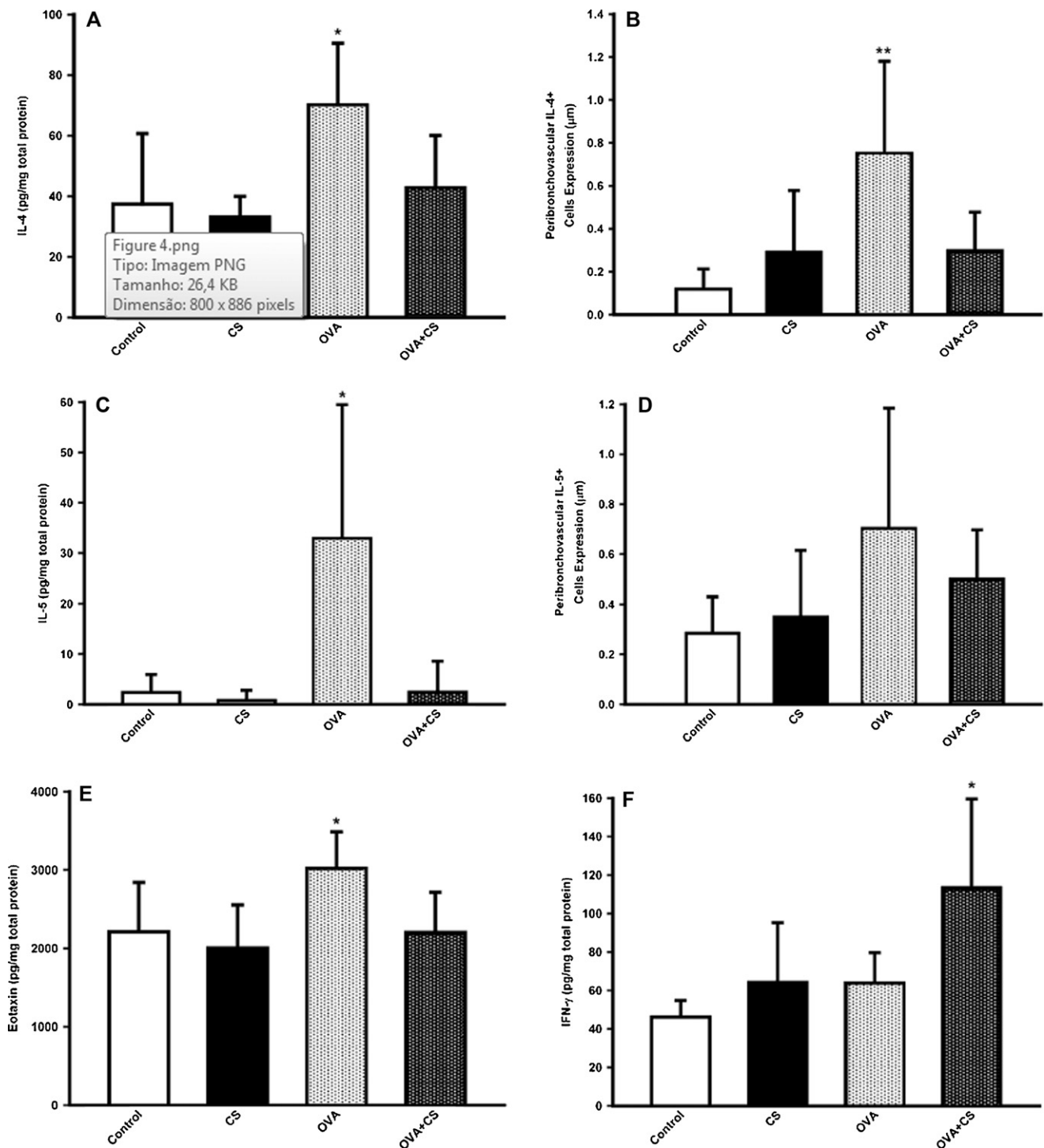


Fig. 4. (A) IL-4 levels from the lung tissue; (B) levels of IL-4-positive cells in the peribronchovascular compartment; (C) IL-5 levels from the lung tissue; (D) levels of IL-5-positive cells in the peribronchovascular compartment. No significant differences were observed; (E) eotaxin levels from the lung tissue; and (F) IFN- γ levels from the lung tissue. The values are presented as the means $\pm$ SD. * $p \leq 0.04$ when compared with all of the other groups; ** $p = 0.01$ compared with the control group.

contrast, an increase in IFN- γ levels in the lung tissue was observed in the OVA + CS group when compared with all of the other groups ($p = 0.001$) (Fig. 4F).

Fig. 5 shows a panel with the levels of IL-10 measured in the Bio-Plex assay and the numbers of IL-10-positive cells in the bronchial epithelium (Fig. 5A and B, respectively). There was an increase in IL-10 levels in the CS, OVA and OVA + CS groups, with the OVA + CS group significantly different from all of the other groups ($p = 0.001$). The CS and OVA groups also showed significant differences

compared with the Control group ($p < 0.05$) (Fig. 5A). The abundance of IL-10-positive cells was also increased in the groups exposed to cigarette smoke when compared with the Control group ($p < 0.05$) (Fig. 5B).

3.4. CS induces airway remodeling in OVA challenged mice

Exposure to ovalbumin resulted in a non-significant increase in collagen fiber content in the peribronchovascular area ($p = 0.06$

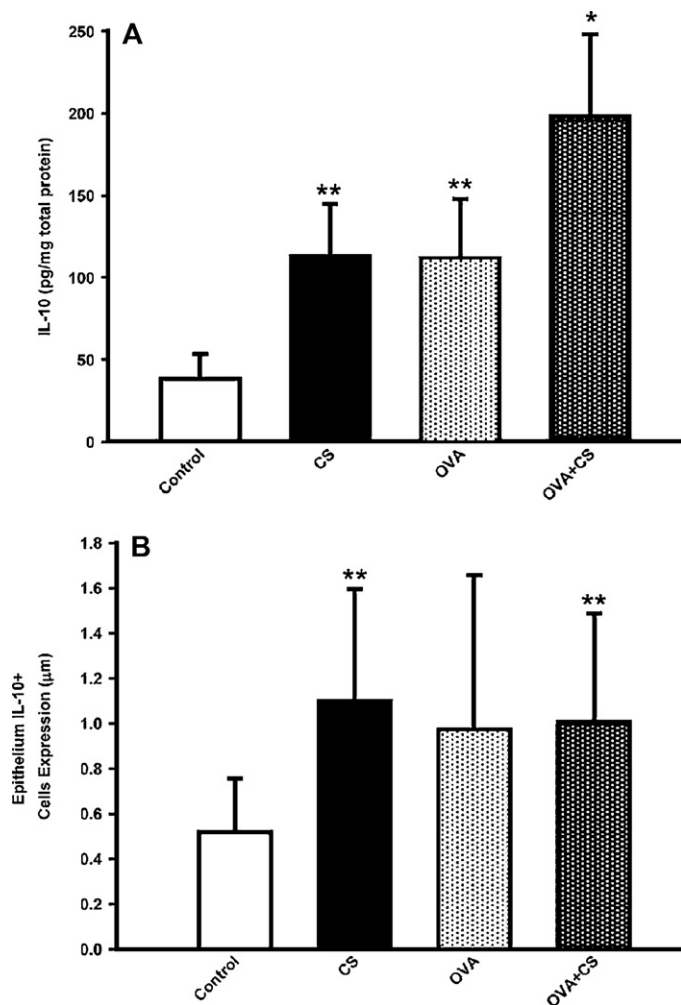


Fig. 5. (A) IL-10 levels from the lung tissue; and (B) levels of IL-10-positive cells in the airway epithelium. The values are presented as the means $\pm$ SD. * $p < 0.001$ when compared with all of the other groups; ** $p < 0.05$ when compared with the control group.

compared with the control group, Fig. 6). Only the OVA + CS group showed a significant increase of collagen fiber content in the peribronchovascular area ($p = 0.001$ compared with the other three groups). Panels A–D show representative photomicrographs of collagen content in the bronchovascular structures in the four experimental groups following staining for collagen fibers.

The OVA + CS group showed a significant increase in the abundance of TGF- β -positive cells in the bronchial epithelium ($p < 0.005$ compared with the Control and CS groups, Fig. 7A). Isolated exposure to either OVA or cigarette smoke did not increase the density of TGF- β -positive cells in the epithelium. In addition, there was a strong correlation between TGF- β -positive epithelial cells and peribronchovascular collagen fiber content (Fig. 6) in the OVA + CS group ($r = 0.91$; $p = 0.01$). The cytokine assay also showed a significant increase in GM-CSF levels in the OVA + CS group compared with all of the other groups ($p = 0.004$) (Fig. 7B). Cigarette smoke exposure also increased VEGF levels, as indicated in Fig. 7C. The OVA + CS group showed a significant difference in VEGF levels compared with the Control and OVA groups ($p = 0.03$). The CS group showed a similar increase in VEGF levels when compared with the control mice ($p = 0.01$).

4. Discussion

In this study, we observed that short-term (3 weeks) exposure to cigarette smoke in mice with pulmonary allergic inflammation induced by ovalbumin exposure results in an attenuation of pulmonary inflammation with a concomitant increase in pulmonary remodeling.

We showed that ovalbumin exposure, with or without co-administration of cigarette smoke, results in a comparable, significant increase in IgE (Fig. 2). The heightened response to Mch observed in OVA-exposed mice was abolished by co-exposure to CS (Fig. 3). The pattern of cytokine release was quite distinctive when CS was added to OVA, with increases in IFN- γ (Fig. 4), IL-10 (Fig. 5), TGF- β , GM-CSF and VEGF (Fig. 7). Peribronchovascular collagen deposition (Fig. 6) was also increased by OVA + CS exposure. These findings suggest the dissociation of pulmonary inflammation and remodeling in this experimental model.

We used an experimental model of allergic pulmonary inflammation that induced pulmonary inflammation. Evaluation of the cells in the bronchoalveolar lavage fluid revealed the presence of a substantial increase in eosinophils, lymphocytes and neutrophils (Table 1). Additionally, we observed an increase in total IgE levels in the blood of mice that were exposed to ovalbumin, and the blood levels of IgE were not influenced by exposure to cigarette smoke. Exposure to cigarette smoke was initiated only three weeks after the first intraperitoneal injection of ovalbumin because our goal was to study the influence of cigarette smoke on the pulmonary inflammation induced by exposure to an allergen and not on the sensitization to the allergen. In addition, our purpose was to expose the mice to cigarette smoke for a short period that would not induce pulmonary changes suggestive of chronic bronchitis or pulmonary emphysema.

OVA exposure resulted in higher values of tissue elastance (Htis) compared with the control and CS groups ($p < 0.05$) (Fig. 3A). This difference was not observed in airway resistance (Raw) (Fig. 3C). This finding is not surprising; in this experimental model, inflammation predominantly occurs in the pulmonary tissue around the airways and in the adjacent blood vessels but not in the bronchial wall (Vieira et al., 2007; Arantes-Costa et al., 2008). The increase in the elastance response to methacholine observed in the mice exposed to ovalbumin was observed for tissue elastance (Htis) but not for airway (Raw) or tissue (Gtis) resistance. Exposure to cigarette smoke attenuated the elastance response to methacholine in mice exposed to ovalbumin. This decrease in pulmonary elastance response may be due to the attenuation of pulmonary inflammation and/or the increase in remodeling. The relationship between eosinophilic inflammation and airway and/or pulmonary responsiveness has been well studied both in humans with asthma and in experimental animals with allergic inflammation (Bento and Hershenov, 1998; Chen et al., 2003; Niimi et al., 2003; Palmans et al., 2000). The decrease in the number of eosinophils infiltrating the lungs in response to cigarette smoke exposure possibly influenced the responsiveness to inhaled methacholine (O'Byrne, 1997).

We observed an increase in peribronchovascular collagen fiber content in mice that were exposed to both ovalbumin and cigarette smoke. Palmans et al. (2000) showed the deposition of extracellular matrix components, such as collagen or fibronectin, in the airway walls of sensitized rats subjected to repeated exposures to allergens. This increase in extracellular matrix component deposition may be associated with attenuated airway smooth muscle (ASM) shortening due to stiffening of the airways. Postmortem studies showed that the ASM layer of patients with asthma is thickened. This may result in airway hyperresponsiveness if the contractility of ASM cells remains constant. However, thickening of the ASM layer is partly attributed to the increased deposition of extracellular

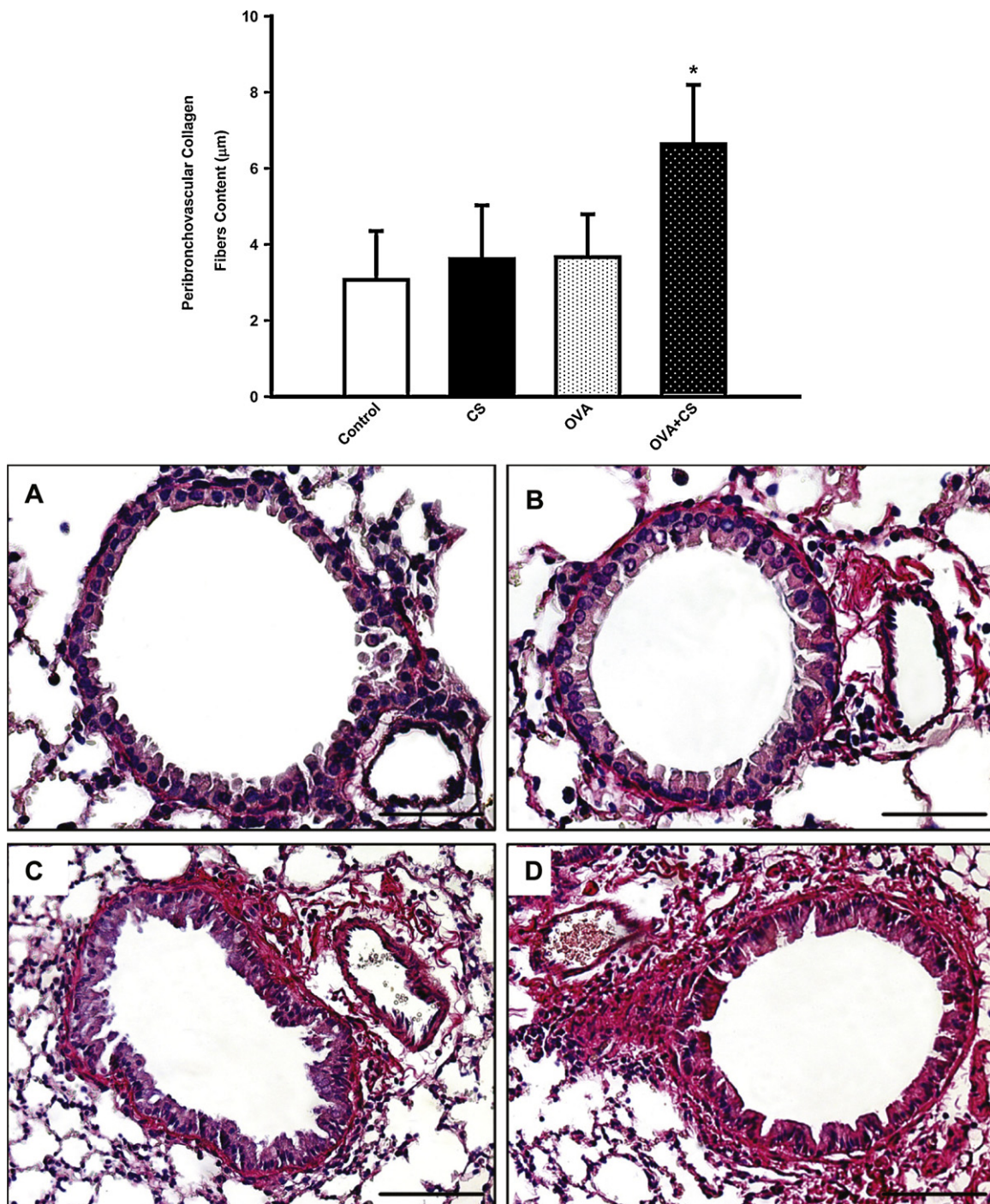


Fig. 6. Collagen fiber content in the peribronchovascular space of the lungs in the four experimental groups. The results are expressed as the area by length (μm). The values are presented as the means $\pm$ SD. * $p = 0.001$ when compared with the other three experimental groups. Panels A–D show representative photomicrographs of collagen fibers stained with picrosirius in all of the groups. A, Control; B, CS; C, OVA and D, OVA + CS.

matrix around individual ASM cells, which may act against ASM shortening (Bento and Hershenon, 1998; Chen et al., 2003; Niimi et al., 2003; Palmans et al., 2000). Thus, it is plausible that the attenuation in tissue elastance observed in the OVA + CS group in this experimental model is related to an increase in collagen fiber content.

Exposure to cigarette smoke can also result in airway remodeling. Churg et al. exposed mice to different periods of cigarette smoke (2 h, 6 h, 24 h, 1 week, 1 month and 6 months) and noted that 2 h after cigarette smoke exposure, there was an approximately six-fold increase in type 1 procollagen gene expression, although this

increase declined over 24 h. Following chronic exposure, there was an approximately eightfold increase in the expression of this gene. The same pattern was observed in the expression of connective tissue growth factor (CTGF) and TGF- β_1 (Churg et al., 2006). However, after 2 h of exposure to cigarette smoke, these changes abate initially and then show a subtle new increase after 1 week, remaining close to the initial values after 6 months of exposure. These data can partially explain our findings because 3 weeks of cigarette smoke exposure alone was not enough to increase collagen fiber content.

We observed a significant increase in TGF- β -positive cells in the bronchial epithelium only in the CS + OVA group after 3 weeks of

cigarette smoke exposure, suggesting an additive or synergic effect of both stimuli (Min et al., 2007). Interestingly, in this group of mice, there was a strong positive correlation between the density of cells in the bronchial epithelium expressing TGF- β and the density of collagen fibers ($r=0.91$; $p=0.01$). Previous studies both in vivo and in vitro revealed a relationship between TGF- β in the bronchial epithelium and lung remodeling with particularly increased expression of types I and III collagen (Kenyon et al., 2003). These findings support the idea that TGF- β can cause lung remodeling even in the absence of detectable inflammation.

In our model, we also observed an increase in GM-CSF and VEGF levels in the OVA + CS group. GM-CSF is a cytokine that influences the proliferation and differentiation of bone marrow cells. The mechanism by which GM-CSF induces collagen synthesis is not completely clear, but it could be due to induction of TGF- β , a known regulator of connective tissue synthesis. GM-CSF was shown to induce TGF- β mRNA expression in vascular smooth muscle and in leiomyoma cells (Brown et al., 2001). These data corroborate our findings, reinforcing the observed increase in the lung remodeling in the OVA + CS group. VEGF is involved in angiogenesis and remodeling and is an autocrine survival factor for epithelial cells (St-Laurent et al., 2009). St-Laurent et al. (2009) studied the bronchial epithelial cells from challenged OVA-sensitized rats and showed an increase in VEGF after 5 days of cigarette smoke extract exposure, and the cigarette smoke-exposed groups also had an increase in VEGF levels. Our data compares favorably with reports from cell-based studies (Brown et al., 2001) that showed an increase in VEGF levels in groups exposed to cigarette smoke and reinforce the increase in pulmonary remodeling in this experimental model.

Cigarette smoke is known to have immunomodulatory properties, but the extent to which smoking cigarettes can alter airway immunity in asthma is not well established (Trimble et al., 2009). Our results showed a significant difference in IFN- γ levels in the OVA + CS group compared with all of the other groups. CS stimuli alone were insufficient to produce an increase in lung IFN- γ levels, suggesting an additional effect of CS on allergic lung inflammation. Although this most likely reflects the toxic effects of cigarette smoke, it is noteworthy that IFN- γ did not abolish, but decreased significantly, the eosinophilic inflammation as expected (Cho et al., 2005; Hofstra et al., 1998; Sopori and Kozak, 1998). In addition, elevated levels of IFN- γ were found in the sputum of patients with asthma, also suggesting that the pathology of asthma could be partially IFN- γ driven (Cho et al., 2005; Sopori and Kozak, 1998).

Many chemical components of cigarette smoke can affect immune function (Sopori and Kozak, 1998; Nouri-Shirazi et al., 2007). One of the most potent and reactive cigarette smoke components is acrolein. Acrolein can influence IL-10, a cytokine with regulatory and anti-inflammatory characteristics capable of inhibiting antigen presentation in macrophages/monocytes (Hristova et al., 2012). This inhibition results in the abrogation of proliferative responses and a decrease in T cell cytokine production (Li et al., 1997, 1999; Li and Holian, 1998; Seymour et al., 1997). This mechanism may be involved in our experimental model because animals exposed to cigarette smoke showed high levels of cytokines in the lung tissue and elevated expression of IL-10 in the bronchial epithelial cells (Kasahara et al., 2008).

In contrast, the expression of IL-4 and IL-5 (Th-2-related cytokines) and eotaxin (an eosinophil chemotactic factor) was increased in peribronchiolar leukocytes, and the total levels of IL-4 and IL-5 were increased in the lung homogenates of OVA-exposed mice, but these levels were not affected by cigarette smoke. The mechanisms by which cigarette smoke attenuates airway eosinophilia are not currently understood. Trimble et al. (2009) observed robust eosinophilic airway inflammation in mice that were exposed to smoke over a sensitization period only, while eosinophilic airway inflammation was attenuated by

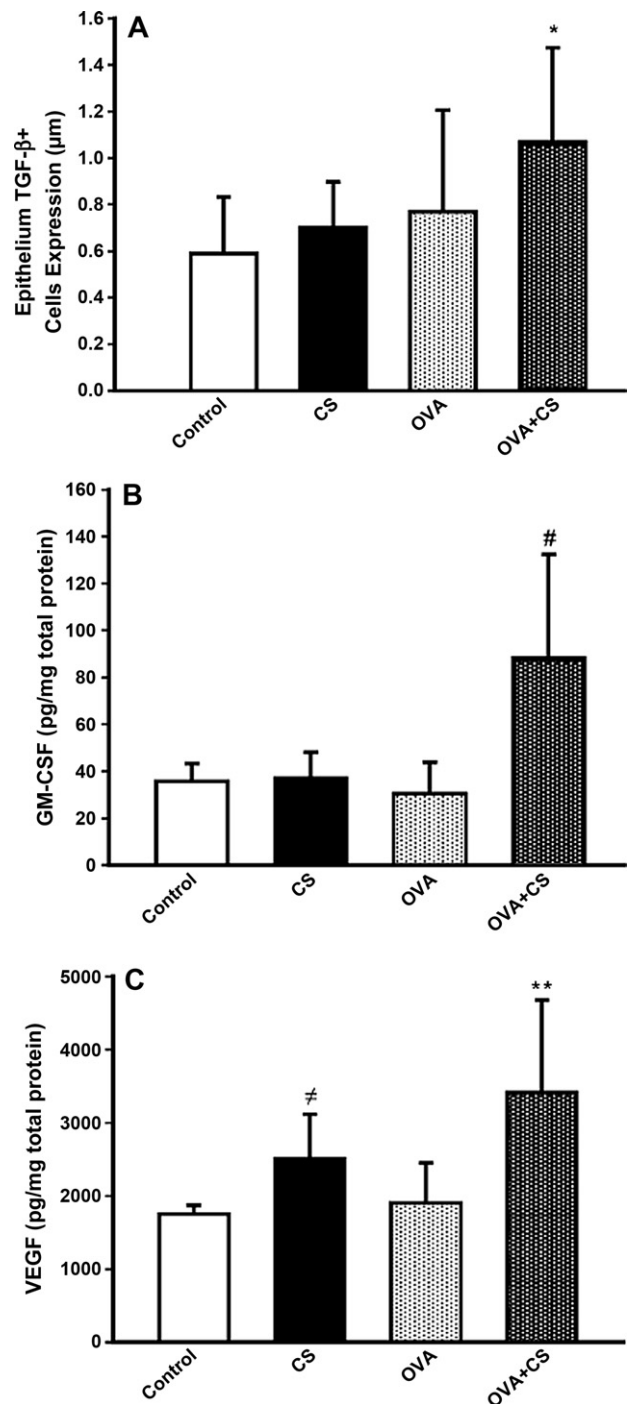


Fig. 7. (A) TGF- β -positive cells in the airway epithelium. Five fields of bronchial epithelium per animal were analyzed. (B) GM-CSF levels from the lung tissue. (C) VEGF levels in the lung tissue. The values are presented as the means $\pm$ SD. * $p < 0.005$ when compared with the control and CS groups; # $p = 0.004$ compared with all of the other groups; ** $p = 0.03$ when compared with the control and OVA groups and *** $p = 0.01$ when compared with the control group.

continuous cigarette smoke exposure (Trimble et al., 2009). These findings imply that cigarette smoke has both adjuvant and anti-inflammatory properties in models of allergic airway inflammation.

Moerloose et al. (2005) observed an exacerbation of the inflammatory responses in animals exposed to smoke (Moerloose et al., 2005). The reasons for these discordant results are unclear. Differences in the experimental approaches may partially explain these results. Seymour et al. (1997) suggested that exposure to

mainstream cigarette smoke or environmental tobacco smoke (ETS) can result in different effects on inflammation and sensitization. In their experiment, they observed that exposure of mice to ETS up-regulated allergic responses to inhaled allergens, while mainstream exposure to cigarette smoke (similar to our experimental model) could act in an opposite way (Seymour et al., 1997).

In our experimental model, we observed an increase in the elastance response to a nebulized methacholine solution in the OVA group. This increase in pulmonary responsiveness was observed when Htis was measured but not when Raw was studied, suggesting that the site of the response was in the lung parenchyma and/or distal airways and not in the central airways. Peták et al. (1997) studied the effects of methacholine-induced bronchoconstriction in rats in response to intravenous (i.v.) versus aerosol administration and suggested that Mch acts on distinct structures when delivered by inhalation or i.v. Mch produces a muscle contraction by stimulating the muscarinic cholinergic receptors (Peták et al., 1997). Sly et al. (1995) investigated the role of the muscarinic receptors in puppies and observed that different receptors may be involved in producing airway and parenchymal constriction in response to inhaled Mch. M3 receptors located on the airway smooth muscle are likely to be responsible for airway responses and may be more easily reached by i.v.-delivered Mch, whereas Mch delivered by the aerosol route must diffuse across the respiratory epithelium before reaching the muscle (Barnes, 1993). In contrast, M1 receptors in the alveolar wall, which are reported to be involved in the parenchymal response (Sly et al., 1995), are likely to be reached more easily by aerosol delivery than by the i.v. route. In our experimental model, the observed increase in the pulmonary response to aerosolized methacholine in the ovalbumin-exposed mice was abolished when there was also cigarette smoke exposure.

Recent studies have shown that airway hyperresponsiveness can be dissociated from cellular inflammation while remaining linked to airway remodeling, and some previous reports also suggested that airway inflammation, lung remodeling and responsiveness may not be directly interrelated (Alcorn et al., 2007; Crimi et al., 1998). Particularly, Alcorn et al. (2007) suggested that attenuated airway remodeling does not impact airway inflammatory responses or airway responsiveness. Corroborating these findings, Kenyon et al. (2003) showed that animals that received a TGF- β_1 instillation had increased the expression of types I and III collagen as well as the total collagen content in the small airways. Notably, there were no signs of inflammation detected in this process. These findings suggest that inflammation and pulmonary remodeling may occur independently (Chapman, 2004; Gauldie et al., 2002; Selman et al., 2001).

In general, these studies demonstrate that airway inflammation, lung remodeling and responsiveness may not be directly interrelated and suggest that the lack of symptoms in some asthmatic patients who smoke (mild smokers) does not imply an absence of any pathologic changes. Bronchial constriction, for example, could be attenuated by an increase of collagen content around airways (Bento and Hershenon, 1998; Chen et al., 2003; Niimi et al., 2003; Palmans et al., 2000).

In summary, in our experimental model, short-term exposure to cigarette smoke in mice with pulmonary allergic inflammation resulted in an attenuation of pulmonary inflammation and responsiveness but led to an increase in lung remodeling.

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