Toll-Like Receptor-9 Agonist Inhibits Airway Inflammation, Remodeling and Hyperreactivity in Mice Exposed to Chronic Environmental Tobacco Smoke and Allergen

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Abstract
Background: As passive environmental tobacco smoke (ETS) exposure in nonsmokers can increase both asthma symptoms and the frequency of asthma exacerbations, we utilized a mouse model, in which ovalbumin (OVA) + ETS induce significantly increased levels of eosinophilic airway inflammation and remodeling compared to either stimulus alone, to determine whether a Toll-like receptor-9 (TLR-9) agonist could reduce levels of airway inflammation, airway remodeling and airway hyperreactivity (AHR). **Methods:** Mice treated with or without a TLR-9 agonist were sensitized to OVA and challenged with OVA + ETS for 1 month. AHR to methacholine was assessed in intubated and ventilated mice. Lung Th2 cytokines and TGF-β_1 were measured by ELISA. Lungs were processed for histology and immunohistology to quantify eosinophils, mucus, peribronchial fibrosis and smooth muscle changes using image analysis. **Results:** Administration of a TLR-9 agonist to mice coexposed to chronic ETS and chronic OVA allergen significantly reduced levels of eosinophilic airway inflammation, mucus production, peribronchial fibrosis, the thickness of the peribronchial smooth muscle lay-er, and AHR. The reduced airway remodeling in mice treated with the TLR-9 agonist was associated with significantly reduced numbers of peribronchial MBP+ and peribronchial TGF-β_1+ cells, and with significantly reduced levels of lung Th2 cytokines [interleukin-5 and interleukin-13] and TGF-β_1. **Conclusion:** These studies demonstrate that TLR-9-based therapies inhibit airway inflammation, remodeling and AHR in mice coexposed to ETS and allergen who exhibit enhanced airway inflammation and remodeling.

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Introduction

Asthma is a disease characterized by airway inflammation and airway hyperreactivity (AHR) [1]. A variety of environmental triggers can aggravate asthma including allergens, viruses, pollutants and tobacco smoke [1]. In terms of tobacco smoke exposure acting as a trigger for asthma, several studies have demonstrated that exposure to either high levels of tobacco smoke in active smokers [2–4] or low levels of tobacco smoke exposure in nonsmokers passively exposed to environmental tobacco smoke (ETS) [5–7] are associated with adverse asthma outcomes including increased prevalence of asthma, increased severity of asthma symptoms, increased frequen-

cy of asthma medication use and increased emergency room visits by asthmatic children. The importance of ETS exposure to asthma is further suggested from recent gene association studies demonstrating a link between a region on chromosome 17q21, combined with ETS, and asthma [8]. Experimental ETS challenge studies in humans also indicate that such exposure has adverse effects on airflow and/or airway responsiveness in asthma [9, 10]. Thus, to reduce the adverse impact of ETS on asthma requires strategies that include reducing the number of smokers and, consequently, of ETS-exposed asthmatics. However, in the USA, although 45% of smokers each year make an attempt to quit, less than 5% of the general population are successful [11]. As up to 68% of nonsmoking children with asthma in the inner cities of the USA are also exposed to ETS as assessed by salivary cotinine levels [7], more immediate strategies are needed to reduce adverse asthma outcomes in ETS-exposed asthmatics in addition to the long-term strategy of reducing the number of smokers.

Studies in mouse models suggest a potential immunologic mechanism for the interaction of ETS and allergen resulting in adverse asthma outcomes. For example, studies by our group [12] and others [13, 14] have demonstrated that exposure of mice to the combination of ETS and ovalbumin (OVA) allergen induces significantly higher levels of Th2 cytokines, eosinophilic airway inflammation, mucus expression, peribronchial fibrosis, thickness of the smooth muscle layer, and AHR compared to levels induced by exposure to either OVA alone or ETS alone. As recent studies suggest that inhaled and oral corticosteroids, currently our most effective anti-inflammatory therapies in asthma, are not as effective in asthmatics who smoke [15–18], there is a need to identify novel therapeutic interventions that inhibit Th2 immune responses both in asthmatics exposed and not exposed to ETS. We have previously demonstrated that administration of a Toll-like receptor-9 (TLR-9) ligand [i.e. immunostimulatory sequences (ISS) of DNA containing a CpG motif] inhibits Th2 cytokine responses [19], eosinophilic airway inflammation [19, 20], airway remodeling [21–24] and AHR [19] in mouse models of asthma. In addition, conjugation of the TLR-9 ligand to the major ragweed allergen Amb a 1 significantly reduces seasonal ragweed rhinitis symptoms in ragweed-allergic human subjects [25]. Based on the ability of TLR-9 ligands to inhibit Th2 cytokine responses [19], as well as on our demonstration that mice coexposed to ETS and OVA allergen have enhanced Th2 cytokine responses [12], we have investigated in the present study whether a TLR-9 ligand would be effective in reducing the enhanced airway inflammation, remodeling and AHR in mice induced by chronic coexposure to ETS and allergen.

Methods

Therapeutic Intervention with TLR-9 Ligand in Chronic ETS + OVA-Exposed Mice
In this study we have examined whether administration of a TLR-9 ligand (i.e. ISS) inhibits airway inflammation, airway remodeling and AHR in mice exposed to chronic ETS in combination with chronic OVA allergen for 1 month. Different groups of 8- to 10-week-old BALB/c mice (12 mice/group; The Jackson Laboratory, Bar Harbor, Me., USA) were chronically exposed to ETS as well as to either no OVA, OVA or OVA + ISS. As controls we also included mice not exposed to ETS (no OVA and OVA). The results of an OVA + corticosteroid intervention in ETS + OVA-exposed mice are reported elsewhere [26], and the present study focuses on the effect of ISS on airway inflammation, airway remodeling, and AHR in ETS-exposed mice. Both intervention studies (ISS or corticosteroid) used the same control groups to limit the number of control mice required for these experiments. The group of ETS-exposed mice that received the OVA + ISS were administered intraperitoneally endotoxin-free phosphorothioate ISS-oligodeoxynucleotides (5′-TGACTGTGAA CGTT CG AGA-TGA-3′; Trilink, San Diego, Calif., USA; 100 μg in 100 μl of sterile, endotoxin-free PBS), starting 1 day before the first intranasal OVA challenge and then continued every other week for the duration of the 1-month period of combined ETS exposure and twice-weekly OVA challenges. Previous studies in our laboratory have demonstrated that ISS inhibits OVA-induced eosinophilic inflammation, airway remodeling and AHR when administered 1 day before OVA challenge [19, 21–24], and that this inhibitory effect lasts at least 4 weeks [27].

Chronic ETS + Chronic OVA Exposure
We have previously demonstrated that chronic ETS exposure alone does not increase airway inflammation, airway remodeling or AHR in mice [12]. In contrast, coexposure of mice to chronic ETS and chronic OVA allergen significantly increases levels of eosinophilic inflammation, airway remodeling and AHR as compared to mice exposed to chronic OVA allergen with no ETS exposure [12].
Chronic ETS Exposure (fig. 1). Three groups of mice (no OVA, OVA and OVA + ISS) were exposed to chronic ETS (side-stream smoke from 6 cigarettes/day each administered over approximately 5 min with a 15-min break between cigarettes, 5 days/week) generated by burning 2R4F reference cigarettes (2.45 mg nicotine/cigarette; Tobacco Research Institute, University of Kentucky, Lexington, Ky., USA) using a smoking machine (McChesney-Jaeger CSM-SSM Single Cigarette Machine, CH Technologies USA, Inc., Westwood, N.J., USA) regulated by programmable controls provided with JASPER Windows 9x/2000 software over RS-232 communication ports (CH Technologies USA, Inc.) as previously described in this laboratory [12]. Each smoldering cigarette is puffed for approximately 2 s, once every 25 s, for a total of 12 puffs/cigarette, at a flow rate of 5 liters/min. The outflow from the smoking machine was adjusted to mimic an exposure to
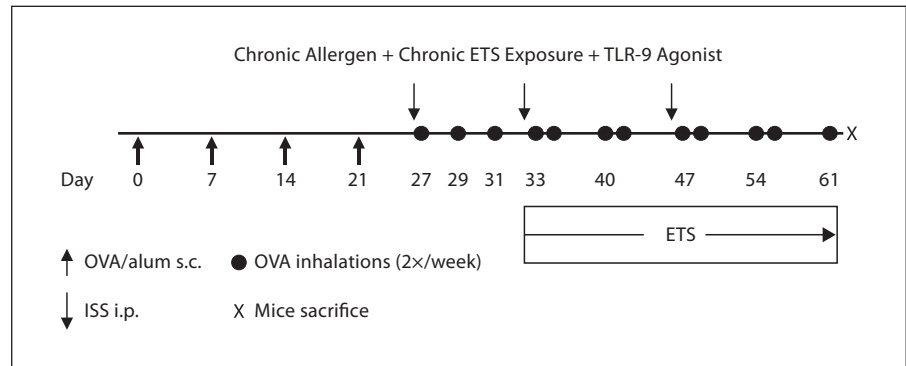
Fig. 1. Mice were immunized s.c. on days 0, 7, 14 and 21 with OVA (arrows pointing up). Intranasal OVA challenges were administered on days 27, 29 and 31, and then repeated twice a week for 1 month. Different groups of mice were either administered ETS alone or ETS in combination with OVA challenges. ETS was started on day 33 (after mice had been sensitized and challenged on 3 occasions with intranasal OVA). The ETS was continued 5 days/week for 1 month. ISS was administered on 3 occasions i.p. (arrows pointing down) starting on day 26 with repeat doses on days 32 and 46. The mice were sacrificed 24 h after the final OVA challenge on day 60 and BAL fluid and lungs were analyzed.

ETS by producing a mixture of room air (98%) and mainstream smoke (2%). The mice were exposed to the ETS in a 12-port, nose-only, directed flow inhalation exposure system (Jaeger-NYU 12 port). Nose ports were monitored for total suspended particulates which we have previously reported to be $173 \pm 5.3 \ \mu g/m^3$ using a gravimetric method [12]. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committee.

Chronic OVA Protocol (fig. 1). In these studies, mice were immunized subcutaneously on days 0, 7, 14 and 21 with 25 μg of OVA (OVA, grade V; Sigma Chemicals, St. Louis, Mo., USA) adsorbed to 1 mg of alum (Aldrich) in 200 μl normal saline as previously described [28]. OVA-challenged mice received intranasal OVA challenges on days 27, 29 and 31 under isoflurane (Vedco, Inc., St. Joseph, Mo., USA) anesthesia, which were then repeated twice a week for 1 month. The no-OVA age- and sex-matched control mice were sensitized but not challenged with OVA during the 1-month study. The groups of mice that were exposed to ETS had their first ETS exposure on day 33 after they had been sensitized with OVA subcutaneously, and received intranasal OVA challenges on days 27, 29 and 31 as previously described in this laboratory [28]. Chronic ETS was continued daily for the subsequent 1-month period of twice-weekly intranasal OVA challenges.

Processing of Lungs for Immunohistology

The mice were sacrificed 24 h after the final chronic OVA and/or chronic ETS challenge and bronchoalveolar lavage (BAL) fluid and lungs were analyzed as previously described [28]. The lungs in the different groups of mice were equivalently inflated with an intratracheal injection of a similar volume of 4% paraformaldehyde solution (Sigma Chemicals) to preserve the pulmonary architecture. These lungs were then processed as a batch for either histologic staining or immunostaining under identical conditions. Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification (×20), gain, camera position and background illumination. The quantita-tive histologic and image analysis of all coded slides was performed by research associates blinded to the coding of all the slides.

BAL and Peribronchial Eosinophils. Total BAL eosinophil counts and the number of peribronchial MBP+ cells were quantitated as previously described [28]. In brief, lung sections were processed for MBP immunohistochemistry using an anti-mouse MBP antibody (kindly provided by James Lee, PhD, Mayo Clinic, Scottsdale, Ariz., USA). The number of individual cells staining positive for MBP in the peribronchial space was counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive for MBP/bronchiole with 150–200 μm of internal diameter. At least 10 bronchioles were counted in each slide.

Mucus. The number of PAS-positive and PAS-negative airway epithelial cells in individual bronchioles were counted as previously described in this laboratory [28]. At least 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS+ cells/bronchiole which is calculated from the number of PAS+ epithelial cells/bronchus divided by the total number of epithelial cells of each bronchiole.

Peribronchial Fibrosis. The area of peribronchial trichrome staining in the paraffin-embedded lungs was outlined and quantified using a light microscope (Leica DMLS; Leica Microsystems, Inc., New York, N.Y., USA) attached to an image analysis system (Image-Pro Plus; Media Cybernetics, Bethesda, Md., USA) as previously described [28]. Results are expressed as the area of trichrome staining/μm length of the basement membrane of bronchioles 150–200 μm of internal diameter.

Peribronchial TGF-β_1+ Cells. The number of peribronchial cells expressing TGF-β_1 were assessed in lung sections processed for immunohistochemistry using an anti-TGF-β_1 primary antibody (Santa Cruz, Calif., USA), the immunoperoxidase method and image analysis quantitation as previously described [28]. Results are expressed as the number of TGF-β_1+ cells/bronchus [28].
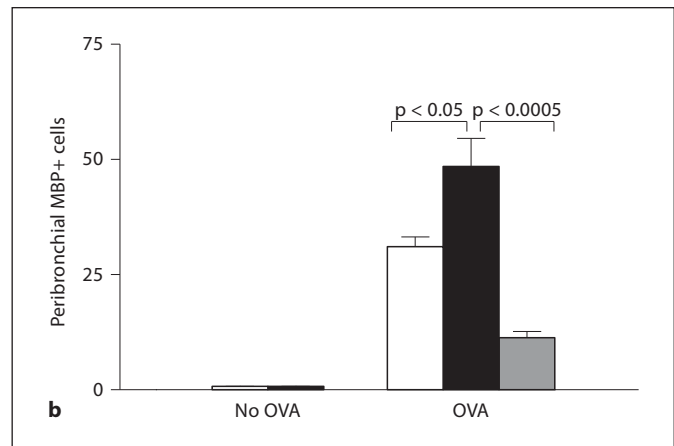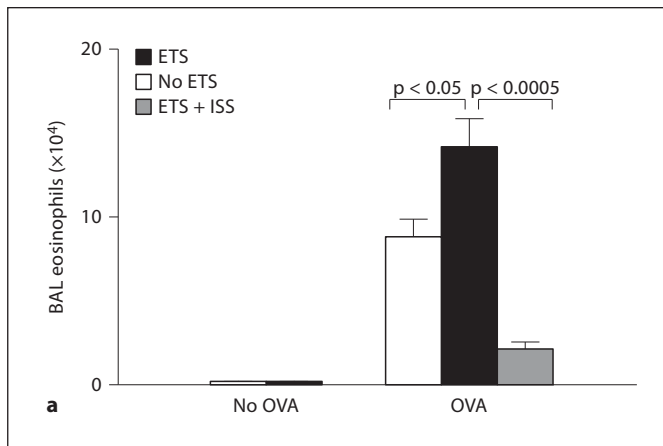
Fig. 2. Eosinophils were quantitated by either Wright-Giemsa staining in BAL fluid (**a**) or by immunostaining lung sections with anti-MBP antibody (**b**). In ETS-exposed mice challenged with OVA, ISS significantly reduced the number of BAL eosino- phils ($p < 0.0005$; ETS + OVA + ISS vs. ETS + OVA) (**a**) and peribronchial eosinophils ($p < 0.0005$; ETS + OVA + ISS vs. ETS + OVA) (**b**).

Thickness of the Peribronchial Smooth Muscle Layer. The thickness of the airway smooth muscle layer (the transverse diameter) was measured from the innermost aspect to the outermost aspect of the smooth muscle layer [28]. The smooth muscle layer thickness in at least 10 bronchioles of similar size (150–200 μm) was calculated on each slide. Lung sections were also immunostained with an anti-α-smooth muscle actin primary antibody (Sigma-Aldrich). The area of α-smooth muscle actin staining was outlined and quantified using a light microscope attached to an image analysis system as previously described [28]. Results are expressed as the area of α-smooth muscle actin staining/μm length of the basement membrane of bronchioles 150–200 μm of internal diameter.

Airway Hyperreactivity. AHR to methacholine (Mch) was assessed 24 h after the final chronic OVA and/or chronic ETS challenge (after 1 month of repetitive OVA ± ETS challenges) in intubated and ventilated mice (flexiVent ventilator; Scireq, Montreal, Que., Canada) as previously described in this laboratory [29]. The frequency-independent airway resistance (R_{aw}) was determined in mice exposed to nebulized PBS and Mch (3, 24 and 48 mg/ml) [29]. In addition to measuring R_{aw}, the Scireq software also recorded tissue elastance (cm H_2O·s/ml) and compliance (ml/cm H_2O).

Lung Levels of Th2 Cytokines and TGF-β_1
Levels of Th2 cytokines (IL-5, IL-13) and TGF-β_1 were measured in BAL by ELISA (R&D Systems, Inc., Minneapolis, Minn., USA). The IL-5 assay has a sensitivity of 15 pg/ml while the IL-13 and TGF-β_1 assays each have a sensitivity of 31 pg/ml.

Percentage Reduction in Inflammation and Remodeling in Response to ISS Therapy
To calculate the percentage reduction in individual indices of airway inflammation and remodeling in response to ISS therapy, the absolute increase in each of these indices in response to OVA + ETS was calculated according to the formula: (OVA + ETS) – (no OVA + ETS). This value is the maximum increase induced by OVA + ETS above baseline values. The reduction of this value induced by ISS therapy was calculated as a percentage.

Statistical Analysis
Results in the different groups of mice were compared by ANOVA using the nonparametric Kruskal-Wallis test followed by posttesting using Dunn's multiple comparison of means. All results are presented as mean ± SEM. A statistical software package (GraphPad Prism; GraphPad Software, San Diego, Calif., USA) was used for the analysis. $p < 0.05$ was considered statistically significant.

Results

Effect of ETS on OVA-Induced Airway Inflammation, Airway Remodeling and AHR
Exposure of mice to chronic ETS alone did not induce an increase in BAL eosinophils (fig. 2a), MBP+ peribronchial eosinophils (fig. 2b), TGF-β_1+ cells (fig. 3a), peribronchial fibrosis (fig. 3b, 4), thickness of the peribronchial smooth muscle layer (fig. 5), AHR (fig. 6a) and mucus production (fig. 7) compared to non-ETS-exposed mice as previously reported in this laboratory [26]. In contrast, chronic ETS in combination with chronic OVA allergen significantly increased all the indices of airway inflammation (fig. 2), airway remodeling (fig. 3b, 4, 5) and AHR (fig. 6a) compared to chronic OVA allergen alone as previously demonstrated in this laboratory [26].
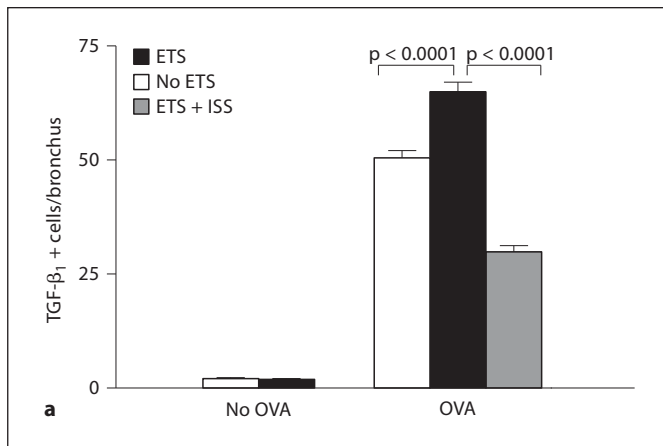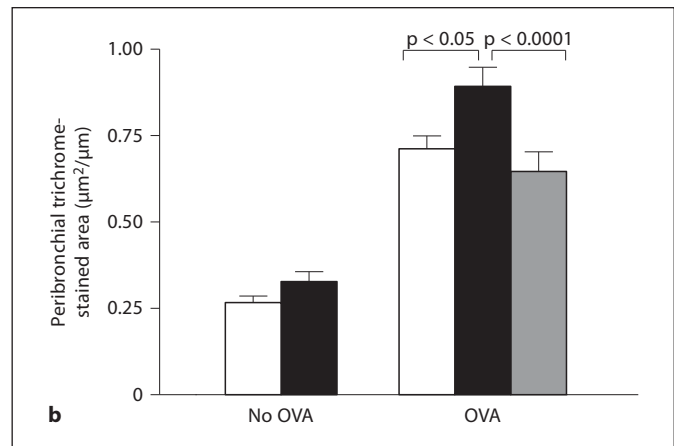
Fig. 3. a The number of peribronchial cells immunostaining positive for TGF-β_1 in mouse lungs was quantitated by image analysis. In ETS-exposed mice challenged with OVA, ISS significantly reduced the number of peribronchial cells immunostaining positive for TGF-β_1 (p < 0.0001; ETS + OVA + ISS vs. ETS + OVA).

b The area of peribronchial trichrome staining in mouse lungs was quantitated in $\mu m^2/\mu m$ length of bronchus by image analysis. In ETS-exposed mice challenged with OVA, ISS significantly reduced the area of peribronchial trichrome staining (p < 0.0001; ETS + OVA + ISS vs. ETS + OVA).

Effect of TLR-9 Ligand on ETS + OVA-Induced Eosinophilic Airway Inflammation

Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced levels of BAL eosinophils by approximately 85% compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.0005) (fig. 2a). Similarly, administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced levels of peribronchial MBP+ eosinophils by approximately 78% compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.0005) (fig. 2b).

Effect of TLR-9 Ligand on ETS + OVA-Induced Peribronchial TGF-β_1+ Cells

Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced the number of peribronchial TGF-β_1+ cells by approximately 72% compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.0001) (fig. 3a).

Effect of TLR-9 Ligand on ETS + OVA-Induced Peribronchial Fibrosis

Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced the area of peribronchial trichrome staining by approximately 44% compared to chronic ETS + OVA allergen-challenged mice

that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.0001) (fig. 3b, 4).

Effect of TLR-9 Ligand on ETS + OVA-Induced Thickness of Peribronchial Smooth Muscle Layer

Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced the thickness of the peribronchial smooth muscle layer by approximately 53% compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.0001) (fig. 5a).

In addition to measuring the thickness of the smooth muscle layer, we also determined the area of peribronchial α-smooth muscle actin immunostaining. Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced the area of peribronchial α-smooth muscle actin immunostaining by approximately 49% compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.0001) (fig. 5b).

Effect of TLR-9 Ligand on ETS + OVA-Induced AHR

Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced AHR compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; Mch 48 mg/ml; p = 0.04) (fig. 6a). Administration of ISS also reduced tissue elastance in OVA + ETS-challenged mice (p < 0.05; Mch 48 mg/ml; ETS + OVA + ISS vs. ETS + OVA)
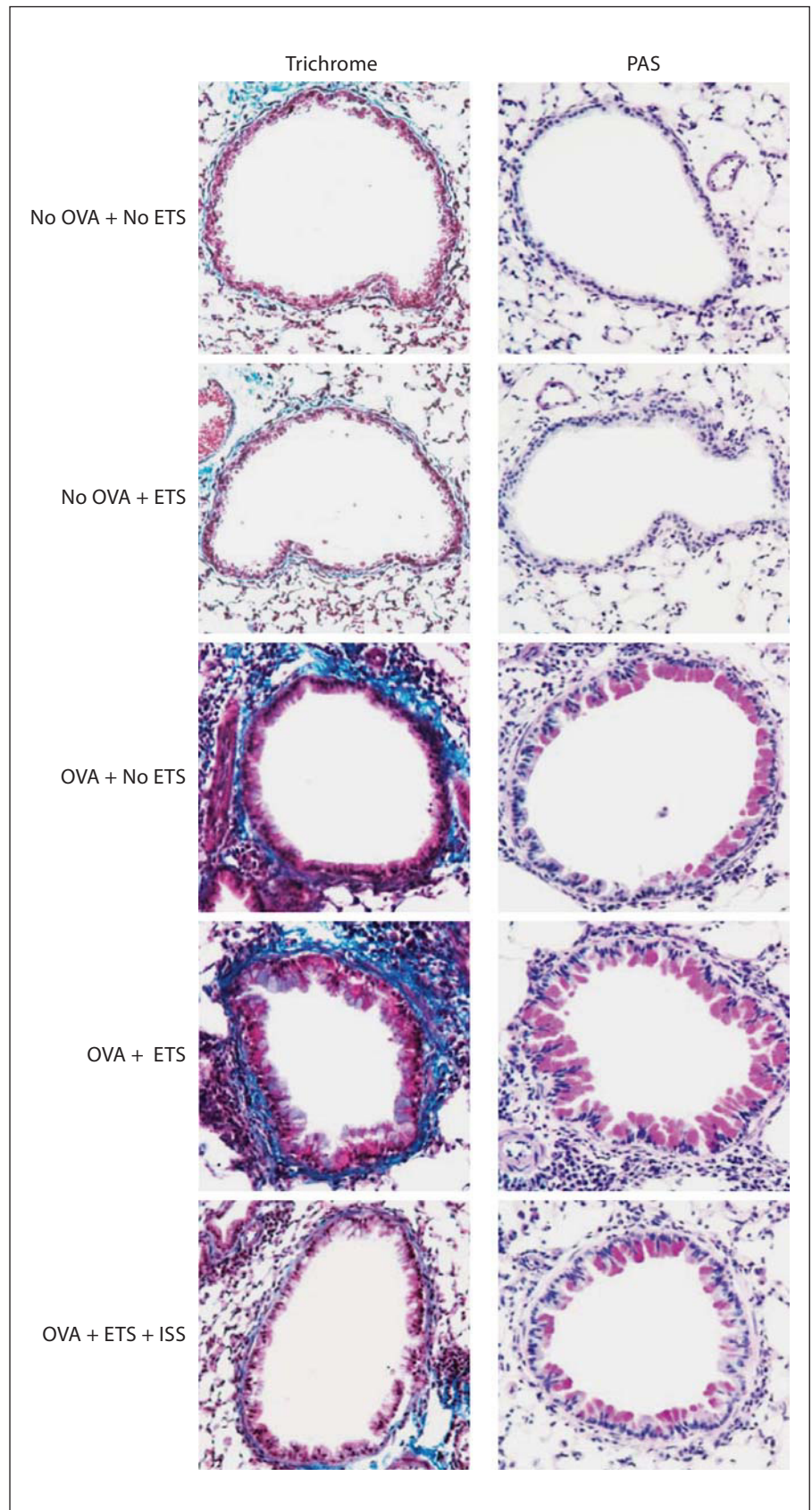
Fig. 4. Lungs from the 5 groups of mice (no OVA, no OVA + ETS, OVA, OVA + ETS, OVA + ETS + ISS) were processed for trichrome staining to detect peribronchial fibrosis (blue) and for PAS staining to detect epithelial mucus expression.
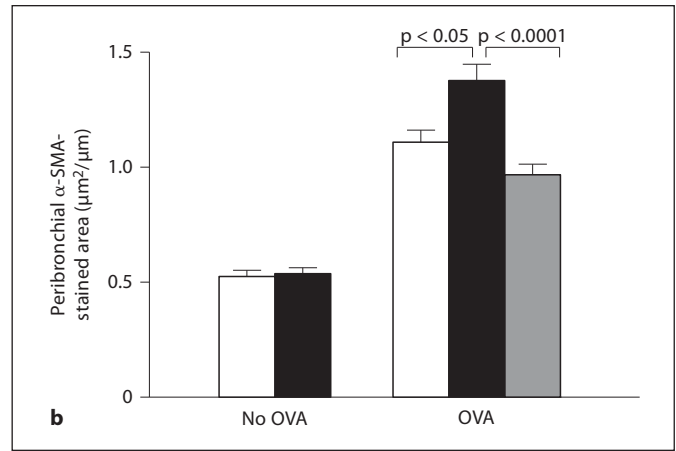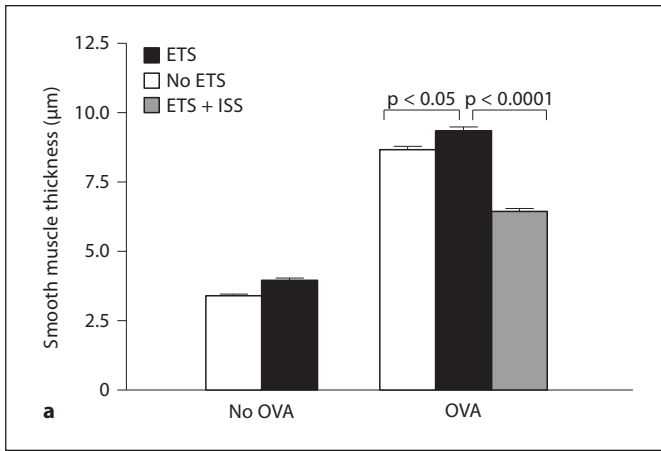
Fig. 5. a The thickness of peribronchial smooth muscle layer was assessed by image analysis. **b** The area of the peribronchial region immunostaining positive with α-smooth muscle actin antibody was quantitated by image analysis ($\mu m^2/\mu m$ length of the basement membrane of the bronchus). In ETS-exposed mice challenged with OVA, ISS significantly reduced both the thickness of the peribronchial smooth muscle layer ($p < 0.0001$; ETS + OVA + ISS vs. ETS + OVA) (**a**) and the area of the peribronchial region immunostaining positive with α-smooth muscle actin antibody ($p < 0.0001$; ETS + OVA + ISS vs. ETS + OVA) (**b**). SMA = Smooth muscle actin.
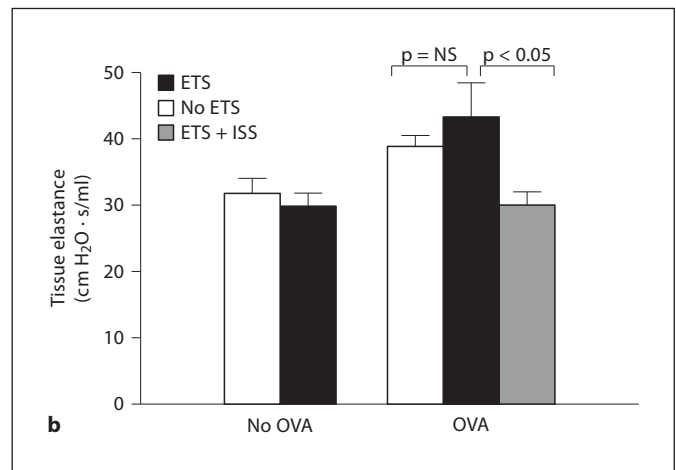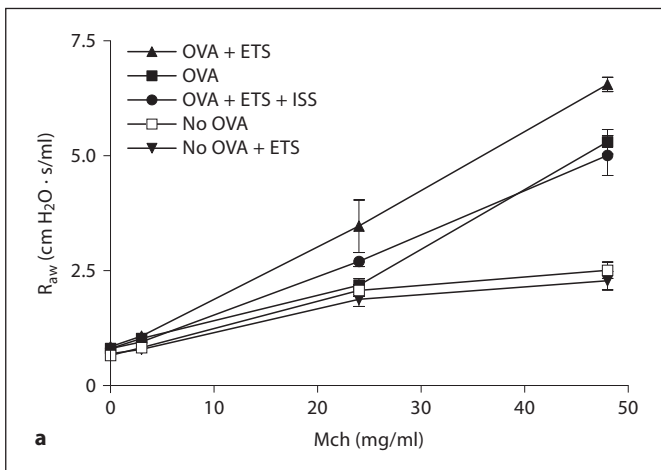

Fig. 6. a AHR to Mch was assessed 24 h after final chronic OVA and/or chronic ETS challenge in intubated and ventilated mice. Results are expressed as R_{aw} in mice exposed to nebulized Mch (3, 24 and 48 mg/ml). In ETS-exposed mice challenged with OVA, ISS significantly reduced AHR ($p < 0.04$; Mch 48 mg/ml; ETS + OVA + ISS vs. ETS + OVA). ISS also reduced tissue elastance in OVA + ETS-challenged mice ($p < 0.05$; Mch 48 mg/ml) (**b**) and improved compliance ($p < 0.001$; Mch 48 mg/ml; ETS + OVA + ISS vs. ETS + OVA) (**c**).
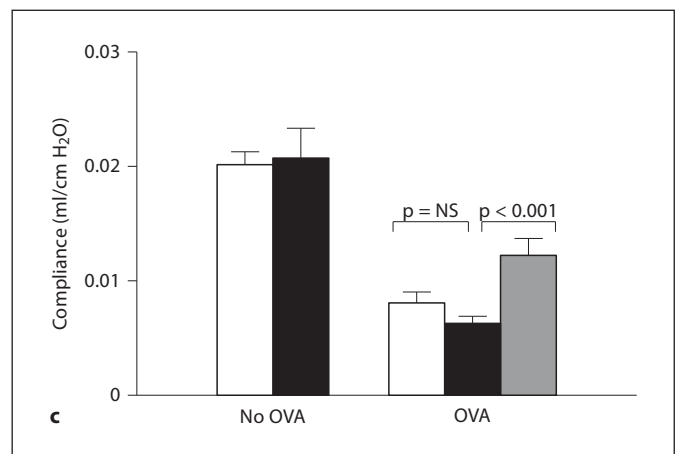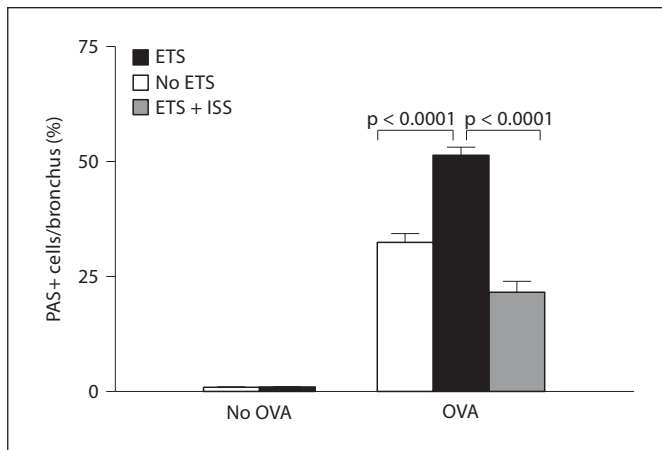
Fig. 7. The percentage of bronchial epithelial cells staining positive for PAS was quantitated by light microscopy. In ETS-exposed mice challenged with OVA, ISS significantly reduced the percentage of PAS+ airway epithelial cells (p < 0.0001; ETS + OVA + ISS vs. ETS + OVA).

(fig. 6b) and improved compliance (p < 0.001; Mch 48 mg/ml; ETS + OVA + ISS vs. ETS + OVA) (fig. 6c).

Effect of TLR-9 Ligand on ETS + OVA-Induced Mucus Expression

Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced levels of mucus expression by approximately 58% compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.0001) (fig. 7).

Effect of TLR-9 Ligand on ETS + OVA-Induced Lung Th2 Cytokines (IL-5, IL-13) and TGF-β_1

Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced levels of BAL IL-5 (fig. 8a), BAL IL-13 (fig. 8b) and TGF-β_1 (fig. 8c) compared to chronic ETS + OVA allergen-challenged mice that did not receive ISS (ETS + OVA vs. ETS + OVA + ISS; p < 0.05).

Discussion

In this study we demonstrated that a TLR-9 agonist (i.e. ISS) is very effective in reducing ETS-enhanced eosinophilic airway inflammation, airway remodeling and AHR in mice exposed to the combination of chronic ETS and chronic OVA allergen. In mice coexposed to ETS and OVA, the TLR-9 agonist inhibited the expression of cyto-

kines that contribute to eosinophilic inflammation (i.e. IL-5), AHR (i.e. IL-13) and airway remodeling (i.e. TGF-β_1). Previous studies from our [12, 26] and other laboratories [13, 14] have demonstrated an enhanced Th2 response to the combination of ETS and allergen as opposed to either stimulus alone. However, the demonstration that a TLR-9 agonist inhibits enhanced airway inflammation, remodeling and AHR in mice exposed to OVA + ETS is novel and most likely due to the ability of TLR-9-based therapies to inhibit Th2 responses to allergen [19], which are enhanced in mice coexposed to allergen and ETS [12, 26] and reduced in those mice administered the TLR-9 ligand. As corticosteroids exhibit a reduced anti-inflammatory effectiveness in smokers [15–18], it is important to identify therapeutic interventions that inhibit Th2 responses in the presence of ETS. In addition to having anti-inflammatory properties, ISS also inhibited airway remodeling in ETS-exposed mice. Studies have shown that smokers with asthma have a more rapid decline in lung function as compared to non-smokers with asthma [2]. Thus, there is also a need to identify novel therapeutic interventions that inhibit airway remodeling in smokers. In mice exposed to allergen + ETS, the increased numbers of cells expressing TGF-β_1 may contribute to airway remodeling as several [30–32], but not all [33], studies in mice in which TGF-β_1 signaling is inhibited demonstrate reduced airway remodeling. Thus, the ability of ISS to significantly reduce levels of lung TGF-β_1 as well as the numbers of peribronchial cells expressing TGF-β_1, such as eosinophils, in mice exposed to ETS + allergen may contribute to a reduction in levels of airway remodeling and airway responsiveness. However, as TLR-9-based therapies influence several different cell types which express TLR-9, the resultant inhibit effects on remodeling and airway responsiveness may, or may not, be linked.

This study extends previous observations that ISS is able to inhibit inflammation, remodeling and AHR in mice exposed to allergen alone [19–24] to demonstrate that ISS can inhibit these asthma outcomes in mice exposed to the combination of ETS and allergen who have enhanced inflammation, remodeling and AHR. As we have previously demonstrated that ISS inhibits these airway inflammation and remodeling outcomes in non-ETS-exposed OVA-challenged mice [21], we did not include a group of ISS-treated, OVA-challenged, non-ETS-exposed mice in this study. Although the lack of a control ISS + OVA group is a limitation of our study, in comparing results of ISS therapy in this ETS + OVA exposure study to previous studies of ISS in non-ETS-exposed
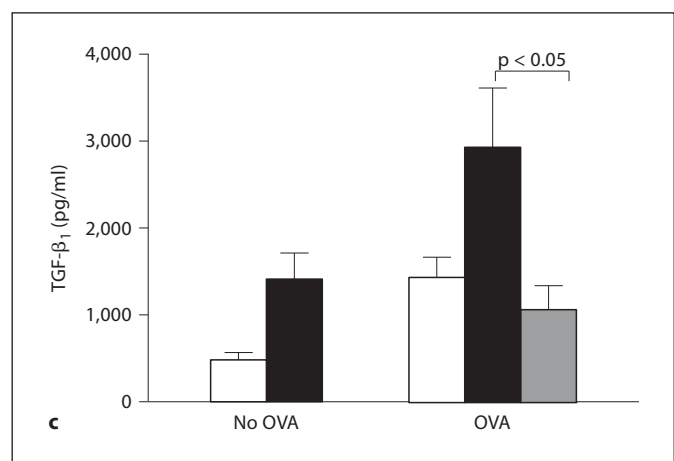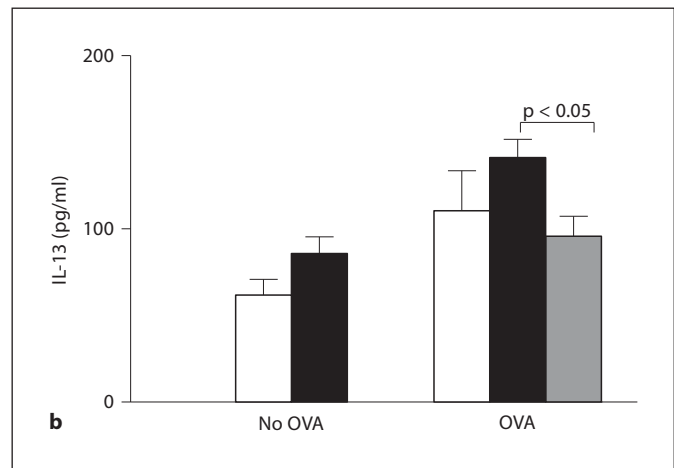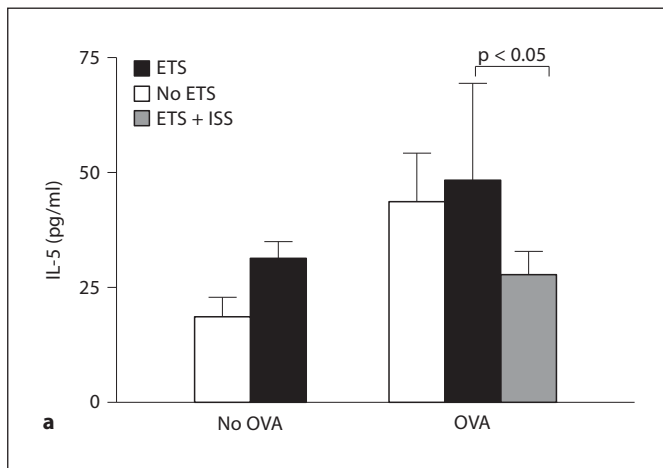
Fig. 8. Levels of Th2 cytokines (IL-5, IL-13) and TGF-β_1 in BAL were measured by ELISA. Administration of ISS to mice exposed to chronic ETS + OVA allergen significantly reduced levels of IL-5 (**a**), IL-13 (**b**) and TFG-β_1 (**c**) compared to chronic ETS + OVA-challenged mice that did not receive ISS (p < 0.05; ETS + OVA vs. ETS + OVA + ISS).

mice, we demonstrated that ISS inhibited airway inflammation and remodeling in mice exposed to ETS + OVA as effectively as in previous studies in which OVA-exposed mice were treated with ISS in the absence of ETS exposure [21]. For example, comparing the levels of inhibition of the indices of airway inflammation and airway remodeling in the current study to those of previous studies by our group [21] demonstrates that ISS induced comparable levels of inhibition of BAL eosinophilia (85 vs. 63%) [21], a reduction in smooth muscle thickness (53 vs. 45%) and a reduction in the number of PAS+ cells (58 vs. 64%). In addition, in the present study ISS reduced OVA + ETS-induced airway inflammation and remodeling to levels significantly below that of untreated mice exposed to OVA alone (OVA + no ETS + no ISS vs. OVA + ETS + ISS), suggesting that ISS was impacting both the OVA as well as the OVA + ETS effect on inflammation and remodeling. These observations, demonstrating the poten-

tial therapeutic utility of ISS in ETS-exposed mice, are potentially important to the large number of asthmatic children and adults exposed to ETS, in whom ETS exposure is associated with adverse asthma outcomes [6, 7, 34]. The large number of asthmatics exposed to ETS has been noted in pediatric and adult studies [6, 7, 34]. For example, approximately 68% of nonsmoking urban children aged 8–14 years had evidence of ETS exposure as assessed by salivary cotinine levels [7] while approximately 29% of nonsmoking adult asthmatics aged 18–50 years in California reported some regular ETS exposure (defined as most days or nights) during an 18-month study period [34]. The ETS-exposed asthmatics had increased asthma severity, increased health care utilization for asthma (emergency department visits, urgent physician visits and hospitalizations) and worse asthma-specific quality of life [34]. The asthmatics who reported cessation of ETS exposure over the 18-month study experienced a reduc-

tion in asthma severity and decreased health care utilization consistent with improved asthma [34]. Conversely, asthmatics with newly initiated significant exposure to ETS over the follow-up period had worsening of asthma severity and asthma-specific quality of life measures [34]. Overall, these studies suggest an important role of ETS in asthma severity and quality of life.

Our study, using a mouse model, has the advantage of being able to accurately provide a well-defined level of exposure to ETS to determine whether ISS is effective in the presence of ETS. The mouse model also demonstrates that this level of ETS exposure has a biological effect in enhancing levels of airway inflammation, mucus, airway remodeling and AHR. The limitation of our study, as with all studies in mouse models, is that it is unknown how the results in the mouse model will translate to the effect of ISS in humans with asthma exposed to ETS. Further studies are also needed to determine whether ISS is effective in inhibiting asthmatic responses at higher doses of tobacco smoke exposure as noted in current smokers. As TLR agonists other than TLR-9 (TLR-2, TLR-4 and TLR-7/8) have inhibitory [35–37] or potentiating effects [38, 39] on inflammation and AHR in OVA models depending upon the timing and route of administration of the TLR agonist, further study is needed to determine the effect of individual TLR agonists other than TLR-9 in mice exposed to OVA + ETS. Prior studies have also demonstrated increased levels of lipopolysaccharide (LPS) in IRF4 cigarettes (approximately 17,800 ng LPS/cigarette) as compared to mainstream smoke (approximately 120 ng LPS/cigarette), and considerably lower levels of LPS in ETS (approximately 18 ng LPS/cigarette) [40]. As the OVA + ETS as well as the OVA + ETS + ISS groups were both exposed to the same ETS (with potentially small amounts of LPS in the ETS), this is very unlikely to account for differences in asthma outcomes observed between these groups of mice.

At present there are limited numbers of studies investigating the therapeutic efficacy of ISS in humans with allergy and asthma. Studies of ISS conjugated to the major ragweed allergen Amb a 1 have demonstrated that in humans with allergic rhinitis the conjugate inhibits Th2 cytokine production by peripheral blood mononuclear cells in vitro [41–43] as well as by cells in the nasal mucosa in response to allergen challenge in vivo [44]. The ISS-Amb a 1 conjugate also inhibits symptoms during the fall ragweed season in subjects with allergic rhinitis [25]. In limited studies in mild asthmatics utilizing an allergen challenge study design, nebulized ISS did not reduce the number of sputum eosinophils or the late-phase response to allergen challenge [45]. Thus, further studies are needed to determine whether ISS, which has demonstrated therapeutic efficacy in mouse [19–24] and primate models of asthma [46], is effective in humans with asthma.

In summary, using a mouse model, our studies demonstrate that ISS significantly reduces levels of eosinophilic airway inflammation, mucus expression, airway remodeling and AHR in mice exposed to the combination of ETS and allergen. However, further human studies are needed to determine whether similar results would be observed in asthmatics treated with ISS who are exposed to ETS. Results from such human studies may be of particular importance to the large number of asthmatics exposed to ETS who have adverse asthma outcomes, as well as to children with the 17q21 gene variant who are at increased risk of developing asthma on exposure to ETS in early childhood [8].

Acknowledgments

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