

Mainstream Cigarette Smoke Exposure Attenuates Airway Immune Inflammatory Responses to Surrogate and Common Environmental Allergens in Mice, Despite Evidence of Increased Systemic Sensitization¹

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The purpose of this study was to investigate the impact of mainstream cigarette smoke exposure (MTS) on allergic sensitization and the development of allergic inflammatory processes. Using two different experimental murine models of allergic airways inflammation, we present evidence that MTS increased cytokine production by splenocytes in response to OVA and ragweed challenge. Paradoxically, MTS exposure resulted in an overall attenuation of the immune inflammatory response, including a dramatic reduction in the number of eosinophils and activated (CD69⁺) and Th2-associated (T1ST2⁺) CD4 T lymphocytes in the lung. Although MTS did not impact circulating levels of OVA-specific IgE and IgG1, we observed a striking reduction in OVA-specific IgG2a production and significantly diminished airway hyperresponsiveness. MTS, therefore, plays a disparate role in the development of allergic responses, inducing a heightened state of allergen-specific sensitization, but dampening local immune inflammatory processes in the lung. *The Journal of Immunology*, 2005, 175: 2834–2842.

Cigarette smoking has been implicated in the development of disease in multiple organ systems and accounts for millions of deaths worldwide annually (1, 2). The majority of tobacco-related deaths are associated with cardiovascular disease, malignancy, and respiratory disorders, such as chronic obstructive pulmonary disease. Furthermore, although there is an association between environmental tobacco smoke (ETS)⁴ (3) and allergic airway diseases, the impact of active smoking on asthma remains controversial despite comparable smoking prevalence between asthmatic and nonasthmatic populations (3).

Epidemiological studies consistently show that ETS is a strong risk factor for the development of asthma. Mounting evidence suggests, for example, that in utero and early childhood exposure to ETS increases the risk of asthma onset and severity in children (4–8). Animal studies have shown that ETS both facilitates allergic sensitization and exacerbates established allergic responses (9–11). The impact of mainstream or active cigarette smoking (MTS)

(4) on asthma, however, is controversial. Many studies have identified smoking as a risk factor for the development of adult-onset asthma (12–15). MTS has been associated with increased serum levels of IgE, skin test positivity, and bronchial hyperresponsiveness toward environmental and occupational allergens (16–18). Active cigarette smoking has also been linked with more severe asthma symptoms, greater need for rescue medication, and worse indices of health status compared with individuals who never smoked (3). Active smoking may even impair responsiveness to therapeutic intervention with corticosteroids (19–21). Other studies, however, have either failed to find such associations (22–24) or have found smokers to be at significantly lower risk of developing asthma compared with nonsmokers and ex-smokers (24, 25). Indeed, Hjern et al. (26) have shown that cigarette smoke may in fact attenuate the development of allergic responses, as indicated by an inverse correlation between the prevalence of allergic asthma or rhinoconjunctivitis and the number of cigarettes smoked. Furthermore, active smokers, although at increased risk of sensitization to house dust mite allergens, are less likely to be sensitized to grass and cat allergens than nonsmokers (27).

To further elucidate the impact of MTS on allergic sensitization and the development of allergic airways inflammation, we exposed mice to MTS before and during sensitization either to a surrogate allergen, OVA (5), or ragweed (RW) (6), a common environmental aeroallergen. We demonstrate that MTS increased cytokine production by splenocytes in response to OVA and RW challenge. Nonetheless, although MTS induced a heightened state of allergen-specific sensitization, cigarette smoke exposure diminished airway hyperresponsiveness and attenuated immune inflammatory responses in the lung.

Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories. Mice were kept in a 12-h light, 12-h dark cycle with unlimited access to food and water. Cages, food, and bedding were autoclaved.

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⁴ Abbreviations used in this paper: ETS, environmental tobacco smoke; BAL, bronchialalveolar lavage; MCh, methacholine; MTS, mainstream tobacco smoke; PAS, periodic acid-Schiff; RSR, respiratory system resistance; RW, ragweed; GM/OVA, GM-CSF/OVA.

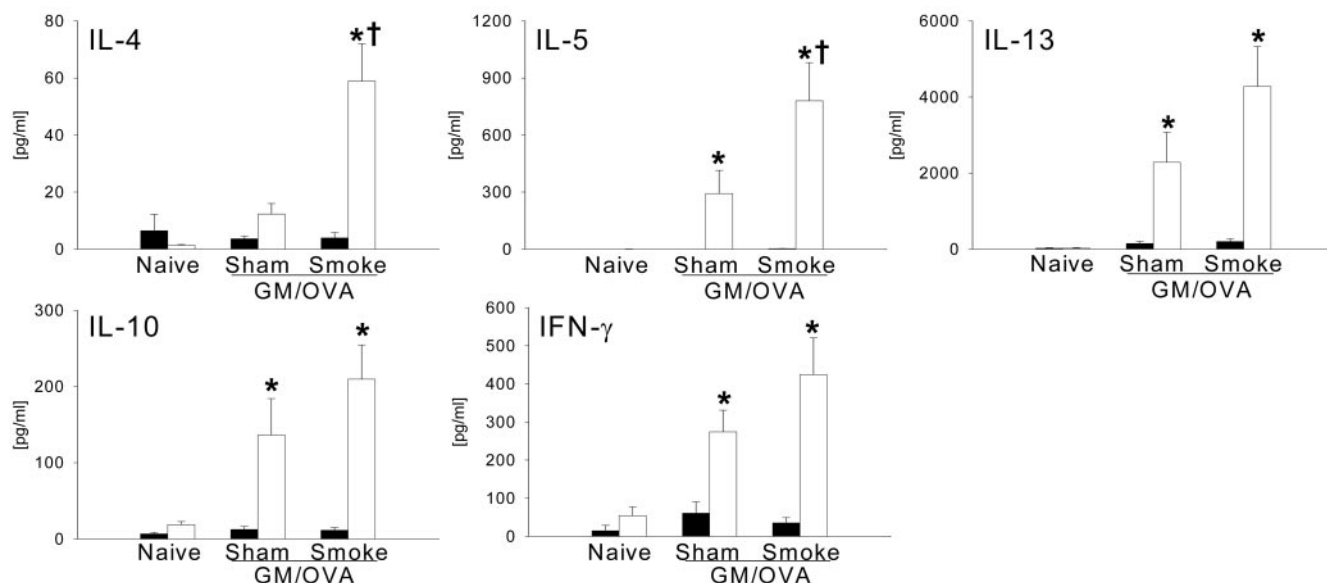


FIGURE 1. In vitro OVA-specific cytokine production. Mice were exposed to either room air (sham) or MTS for 2–3 mo and treated with GM/OVA as outlined in *Materials and Methods*. Splenocytes were harvested 48 h after the final OVA exposure and placed in culture in the presence of either medium alone (■) or OVA (□). Cytokine production was assessed in cell culture supernatants by commercial ELISA. Data are expressed as the mean $\pm$ SEM ($n = 7$ –12). Statistical analysis was performed using one-way ANOVA with the Student-Newman-Keuls method; $p < 0.05$ was considered significant. *, Statistically significant from naive; †, statistically significant compared with sham GM/OVA.

Animal manipulations were conducted in a laminar flow hood by personnel who were gloved, gowned, and masked. All experiments described in this study were approved by the McMaster University animal research ethics board.

Smoke exposure protocol

Mice were exposed to MTS in a smoke exposure system initially developed for guinea pigs (28) and later adapted for mice (29). Mice were exposed to two cigarettes daily (1R3 reference cigarettes; Tobacco and Health Research Institute, University of Kentucky), 5 days/wk. The duration of the exposure protocol is indicated in the figure legends. Sham-exposed animals were placed in restrainers only.

OVA exposure protocol

Mice were subjected to a model of OVA-specific mucosal sensitization that has been described by us in detail previously (30). Briefly, a replication-deficient human type 5 adenoviral construct carrying murine GM-CSF cDNA in the E1 region of the viral genome was delivered intranasally into anesthetized animals 24 h before OVA exposure. Adenoviral/GM-CSF was administered at a dose of 3×10^7 PFU in 30 μ l of PBS vehicle (two 15- μ l instillations, 5 min apart). Mice were exposed to OVA (1%, wt/vol, in 0.9% saline; grade V; Sigma-Aldrich) over a period of 10 consecutive days (or as indicated in the figure legends) in a Plexiglass chamber ($10 \times 15 \times 25$ cm). OVA was administered by aerosolization for 20 min daily using a Bennet twin nebulizer (Puritan-Bennet) at 10 l/min. For in vivo rechallenge, 4 wk after the last OVA exposure, mice were exposed to 1% OVA aerosol for 20 min on 3 consecutive days. Mice were killed 72 h after the final OVA aerosolization.

RW exposure protocol

Mice were exposed to RW protein extract intranasally according to a protocol previously described by Cates et al. (31). Briefly, anesthetized mice were inoculated with 10 μ l of 20 mg/ml RW protein extract (Greer Laboratories) in Coca's buffer (0.085 M NaCl and 0.064 M NaHCO₃, pH 8.1) daily for 7 consecutive days. RW was delivered within 30 min of MTS exposure.

Collection and measurement of specimens

Lungs were removed, tracheas were cannulated (BD Biosciences), and bronchialalveolar lavage (BAL) was obtained by lavaging twice with PBS (250 and 200 μ l). Total cell counts were determined by hemocytometer. BAL supernatants were stored at -20°C until assayed for cytokine levels. Cytospins for differential cell counts were prepared by resuspension of cell

pellets in PBS, followed by cytocentrifugation at $10 \times g$ for 2 min. Cells were stained by Hema 3 (Biochemical Sciences), and differentials were determined by counting at least 300 cells. Standard hemocytological criteria were used to classify mononuclear cells, neutrophils, and eosinophils. Lung tissue was fixed at 20 cm H₂O pressure in 10% formalin, embedded in paraffin, and 3- μ m-thick sections were stained with either H&E or Congo Red, and Periodic acid-Schiff (PAS). To enumerate lung tissue eosinophils in OVA studies, peribronchial and perivascular eosinophils were enumerated by counting the number of Congo Red-staining cells per field (magnification, $\times 200$); at least 10 fields were collected per sample. In RW studies, entire H&E lung sections were scanned using a Coolscan II slide scanner (Nikon). A grid (0.70 mm²) was superimposed over scanned images, and eosinophils were counted in five random squares per tissue under the light microscope. PAS staining was assessed using a semiquantitative method. Airways were examined under light microscopy and assigned a score between 0 and 3 based on the following criteria: 0, no staining; 1, PAS staining $<25\%$ of airway perimeter; 2, PAS staining 25 to $<50\%$ of airway perimeter; and 3, PAS staining $>50\%$ of airway perimeter. Blood was collected by retro-orbital bleeding, and serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C . Samples were stored at -20°C until assayed.

Measurements of cytokines and Igs

ELISA kits for murine IL-4, IL-5, IL-13, IL-10, and IFN- γ were purchased from R&D Systems. The threshold of detection for these systems was 2–7 pg/ml. OVA- and RW-specific Igs were detected using custom developed ELISAs, which were described previously (31, 32).

Splenocyte and lung cell isolation

Spleens were removed and immediately placed on ice in HBSS (Invitrogen Life Technologies). The tissue was triturated through a sterile 70- μ m pore size nylon cell strainer (VWR International) using the plunging end of a sterile 3-ml syringe. The cell suspension was centrifuged at $250 \times g$ for 10 min at 4°C . RBC were lysed with ACK lysis buffer (0.5 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA, pH 7.2–7.4), and the splenocytes were washed twice with HBSS and resuspended in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen Life Technologies).

For isolation of lung cells, lungs were perfused via the right ventricle with 10 ml of warm (37°C) HBSS (calcium and magnesium free) containing 5% FBS and 1% penicillin/streptomycin. The lungs were then cut into small pieces (~ 2 mm in diameter) and shaken at 37°C for 1 h in 15 ml of 150 U/ml collagenase III (Worthington Biochemical) in HBSS. Using a

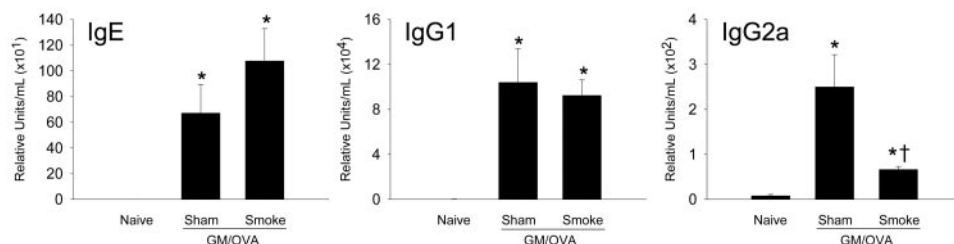


FIGURE 2. OVA-specific Ig production. Mice were exposed to either room air (sham) or MTS for 2–3 mo and treated with GM/OVA as outlined in *Materials and Methods*. OVA-specific IgE, IgG1, and IgG2a were measured in serum by ELISA. Data shown represent the mean $\pm$ SEM ($n = 7$ –12). Statistical analysis was performed using one-way ANOVA with the Student-Newman-Keuls method; $p < 0.05$ was considered significant. *, Statistically significant compared with naive; †, statistically significant compared with sham GM/OVA.

plunger from a 3-ml syringe, the lung pieces were triturated through a 40- μ m pore size nylon cell strainer. Cells were washed twice, and mononuclear cells were isolated by density centrifugation in 30/60% Percoll (Pharmacia Biotech).

Flow cytometry

The following mAbs were selected to study the phenotype of lung mononuclear cells: anti-CD3 (CyChrome-conjugated 145-2C11), anti-CD4 (FITC-conjugated L3T4), anti-CD69 (PE-conjugated H1 2F3), anti-T1ST2 (FITC-conjugated 3E10), anti-CD11c (PE-conjugated HL3), anti-IA^d (biotin-conjugated 39-10-8), and anti-B220 (PE-conjugated RA3-6B2); streptavidin-PerCP (BD Biosciences) was used as a second-step reagent for detection of biotin-labeled Abs. Titration was performed to determine the optimal concentration of each Ab. All Abs were purchased from BD Pharmingen, except anti-T1ST2, which was provided by Millennium Pharmaceuticals. To minimize nonspecific binding, 10^6 cells were incubated with 0.5 μ g of Fc Block (CD16/CD32; BD Pharmingen) on ice for 15 min. Subsequently, cells were stained with first-stage mAbs on ice for 30 min, washed, and then treated with second-stage reagents. Data were collected using a FACScan (BD Biosciences) and analyzed using WIN-MDI software (Scripps Research Institute). A total of 5×10^4 to 1×10^5 events were acquired.

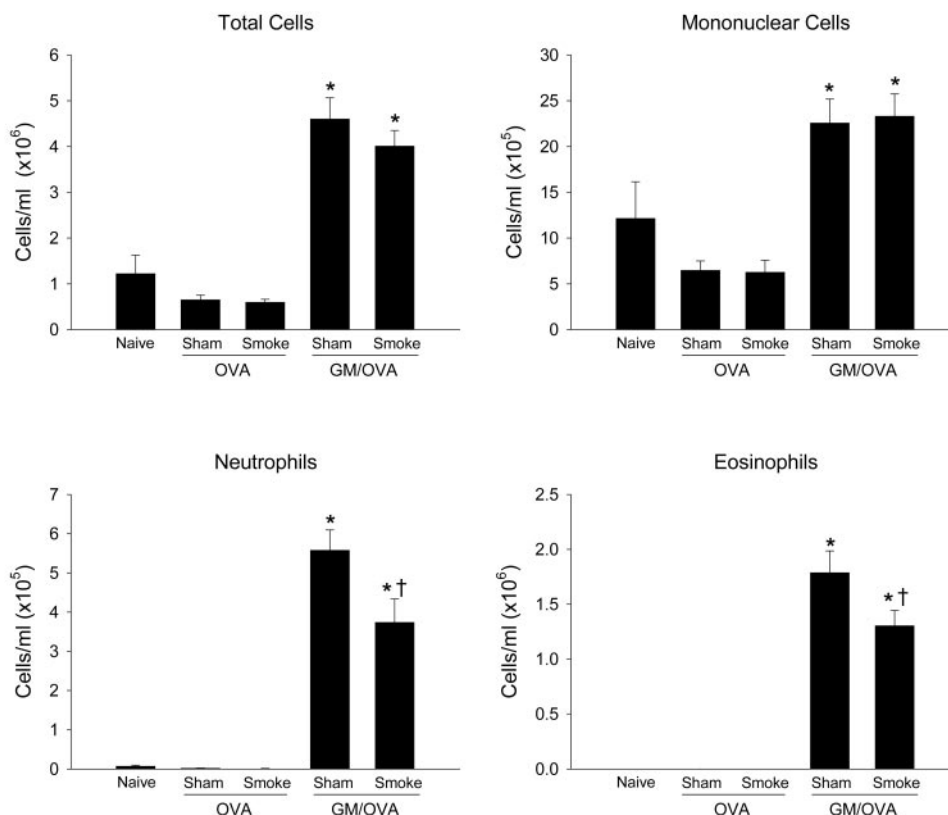
Measurement of airway responsiveness

Airway responsiveness was measured based on the response of total respiratory system resistance (RSR) to increasing doses of methacholine (MCh) injected into the internal jugular vein of Avertin-anesthetized mice as previously described (33–35). Exposed tracheas were cannulated, and a constant inspiratory flow was delivered by mechanical ventilation (RV5; Voltek Enterprises). Pancuronium (0.03 mg/kg i.v.) was used to induce paralysis, thus preventing a respiratory effort during measurement. RSR was measured after consecutive i.v. injections of saline, followed by 10, 33, 100, 330, and 1000 μ g/kg MCh (ACIC), each delivered in a 0.2-ml bolus. Airway responsiveness was evaluated based on the peak RSR measured in the 30 s after the saline and MCh challenges.

Data analysis

Data are expressed as the mean $\pm$ SEM. Statistical interpretation of results is indicated in the figure legends. All statistical analysis was performed using SigmaStat statistical software (version 2.0; SPSS). Differences were considered statistically significant at $p < 0.05$.

FIGURE 3. Inflammatory profile in the BAL of GM/OVA-exposed mice. Mice were exposed to either room air (sham) or MTS for 2–3 mo and given GM/OVA as outlined in *Materials and Methods*. Forty-eight hours after the final OVA exposure, lungs were lavaged, and the numbers of mononuclear cells, neutrophils, and eosinophils were determined. Data shown represent the mean $\pm$ SEM ($n = 7$ –12). Statistical analysis was performed using one-way ANOVA with the Student-Newman-Keuls method; $p < 0.05$ was considered significant. *, Statistically significant compared with naive, sham OVA, and smoke OVA; †, statistically significant compared with sham GM/OVA.



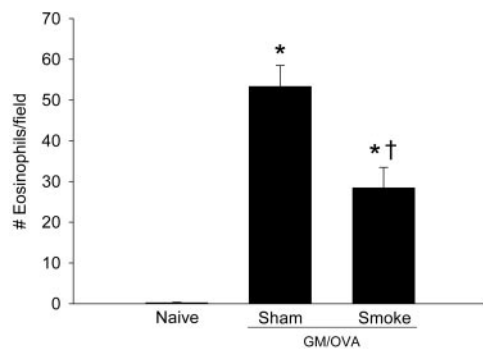


FIGURE 4. Lung tissue eosinophilia in GM/OVA-exposed animals. Mice were exposed to either room air (sham) or MTS for 2–3 mo and given GM/OVA as outlined in *Materials and Methods*. Forty-eight hours after the final OVA exposure, lungs were isolated, sections were stained with Congo Red, and the number of eosinophils were enumerated. Data shown represent the mean $\pm$ SEM ($n = 7$ –12). Statistical analysis was performed using one-way ANOVA with the Student-Newman-Keuls method; $p < 0.05$ was considered significant. *, Statistically significant compared with naive; †, statistically significant compared with sham GM/OVA.

Results

In vitro production of OVA-specific cytokines

To investigate the impact of MTS on allergic sensitization, we investigated *in vitro* production of Ag-specific cytokines by spleen cells. Mice were exposed to cigarette smoke for 2–3 mo and sensitized to OVA (GM-CSF/OVA (OVA)) as outlined in *Materials and Methods*. Mice were killed 48 h after the last OVA exposure, and spleen cells were isolated and placed in culture. OVA-specific cytokines were measured in cell culture supernatants by ELISA. We observed a significant increase in OVA-specific production of IL-4 and IL-5 by splenocytes isolated from MTS-GM/OVA-exposed animals compared with sham-GM/OVA-exposed mice (Fig. 1). Similarly, we observed a trend toward increased production of IL-13, IL-10, and IFN- γ in MTS-GM/OVA-exposed mice compared with sham-GM/OVA-exposed animals, although these values did not reach statistical significance. We observed negligible levels of cytokine production by cells isolated from naive animals.

OVA-specific Ig production

Allergic sensitization leads to increased circulating levels of allergen-specific Igs. To address the impact of MTS on Ab responses,

sham- and MTS-GM/OVA-exposed mice were killed 48 h after the last OVA aerosolization, and serum levels of OVA-specific IgE, IgG1, and IgG2a were assessed. We observed increased levels of OVA-specific Igs in both sham- and MTS-GM/OVA-exposed mice compared with naive animals (Fig. 2). Moreover, although we observed similar levels of OVA-specific IgE and IgG1 in sham- and MTS-GM/OVA-exposed animals, MTS exposure resulted in a 75% reduction in OVA-specific IgG2a production.

OVA-induced airway inflammation

Because we observed increased allergen-specific cytokine responses in cigarette smoke-exposed animals, we next determined the impact of MTS on allergic airways inflammation. Sham- and MTS-GM/OVA-exposed mice were killed 48 h after the last OVA aerosolization and the BAL cellular profile was assessed. GM/OVA exposure resulted in increases in the total number of cells recovered from the BAL of both sham and MTS mice, compared with naive animals and sham and MTS mice given OVA alone (Fig. 3). This change was characterized by an increase in mononuclear cells, neutrophils, and eosinophils. Contrary to our expectation, we observed a significant reduction in both the number (Fig. 3) and the percentage (data not shown) of eosinophils and neutrophils in MTS-GM/OVA-exposed animals compared with sham-GM/OVA-exposed mice.

To investigate whether the impact of MTS exposure on airway eosinophilia was associated with similar changes in the lung, lung tissue sections from sham- and MTS-GM/OVA-exposed mice were stained with Congo Red and perivascular and peribronchial eosinophils were enumerated. We observed an increase in the number of eosinophils in both sham- and MTS-GM/OVA-exposed mice compared with naive animals (Fig. 4). Furthermore, MTS-GM/OVA-exposed mice demonstrated significantly fewer ($\sim 50\%$) tissue eosinophils compared with sham-GM/OVA-exposed animals.

Flow cytometric analysis of the lung tissue

Allergic sensitization and maintenance of allergic inflammatory processes are driven in part by Th2-polarized CD4 T cell responses. Therefore, we next assessed the impact of MTS on the immunological cellular profile in lung tissue. Sham- and MTS-GM/OVA-exposed mice were killed 48 h after the last OVA aerosolization, lung mononuclear cells were isolated, and the percentage and number of dendritic cells (MHCII^{bright}/CD11c^{bright}), B

Table I. Flow cytometric analysis in the lung^a

	Naive	GM/OVA ($\times 10^5$ cells/lung) (%)	
		Sham	Smoke
Dendritic cells			
CD11c ^{bright} /MHCII ^{bright}	0.09 $\pm$ 0.02 (0.7 $\pm$ 0.2)	7.08 $\pm$ 2.41 (2.5 $\pm$ 0.3 ^b)	4.42 $\pm$ 0.94 (3.3 $\pm$ 0.4 ^b)
B cells			
B220 ⁺	1.38 $\pm$ 0.54 (8.2 $\pm$ 0.5)	2.83 $\pm$ 0.81 (1.0 $\pm$ 0.1 ^b)	1.53 $\pm$ 0.37 (1.1 $\pm$ 0.1 ^b)
CD4 T cells			
CD3 ⁺ /CD4 ⁺	1.39 $\pm$ 0.53 (8.3 $\pm$ 1.2)	21.96 $\pm$ 6.08 ^b (7.8 $\pm$ 0.5)	12.22 $\pm$ 3.20 (8.5 $\pm$ 0.4)
CD69 ⁺	0.85 $\pm$ 0.32 (7.7 $\pm$ 3.6)	9.85 $\pm$ 2.88 ^b (44.5 $\pm$ 1.1 ^b)	5.01 $\pm$ 1.19 (41.7 $\pm$ 1.2 ^b)
T1ST2 ⁺	0.45 $\pm$ 0.10 (3.4 $\pm$ 1.4)	2.78 $\pm$ 0.85 (12.7 $\pm$ 1.7 ^b)	0.98 $\pm$ 0.22 (9.8 $\pm$ 0.3 ^b)

^a Mice were exposed to either room air (sham) or MTS for 2–3 mo and administered GM/OVA as outlined in *Materials and Methods*. Forty-eight hours following the last OVA exposure, lung mononuclear cells were isolated and flow cytometric analysis was used to identify dendritic cells (CD11c^{bright}/MHCII^{bright}), B cells (B220⁺), and activated (CD69⁺) and Th2-associated (T1ST2⁺) CD4 T cells (CD4⁺/CD3⁺). Data shown represent mean $\pm$ SEM. Dendritic cells, B cells, and CD4 T cells are expressed as percentages of the total number of mononuclear cells. CD69⁺ and T1ST2⁺ cells are expressed as percentage of cells within the CD4 T cell gate; $n = 3$ for naive (three experiments where three to five lungs were pooled per experiment); $n = 4$ for sham GM/OVA and smoke GM/OVA (one representative experiment of three). Statistical analysis was performed using one-way ANOVA with the Student-Newman-Keuls method; $p < 0.05$.

^b Statistically significant compared to naive.

Table II. Cytokine production in the BAL and serum^a

	Naive (pg/ml)	Sham-GM/OVA (pg/ml)	Smoke-GM/OVA (pg/ml)
BAL			
IL-5	ND	27.3 ± 11.0	54.1 ± 15.0
IL-13	ND	410.0 ± 79.7	608.4 ± 87.4
Eotaxin	ND	59.8 ± 9.4	77.0 ± 8.2
Serum			
IL-13	45.0 ± 25.9	115.1 ± 15.1	152.2 ± 27.9

^a Mice were exposed to either room air (sham) or MTS for 2–3 mo and administered GM/OVA as outlined in *Materials and Methods*. Twenty-four hours following the ninth OVA exposure, lungs were lavaged, serum was obtained, and cytokine production was assessed by ELISA. Data shown represent mean ± SEM; *n* = 3–10. Statistical analysis was performed using one-way ANOVA.

cells (B220⁺), and CD4 T cells (CD3⁺/CD4⁺) were assessed by flow cytometry. We also examined the expression of the activation marker, CD69, and the putative Th2 marker, T1ST2, on CD4 T cells. In three independent experiments we recovered $48.8 \pm 9.6\%$ fewer cells from MTS-GM/OVA-exposed mice compared with sham-GM/OVA-exposed animals. In one representative experiment of three we isolated $14.8 \pm 3.6 \times 10^6$ and $29.1 \pm 8.7 \times 10^6$ cells/lung from MTS-GM/OVA-exposed mice compared with sham-GM/OVA-exposed animals. Therefore, although we observed a similar increase in the percentage of dendritic cells and activated (CD69⁺) and Th2-associated (T1ST2⁺) CD4 T cells in sham- and MTS-GM/OVA-exposed mice compared with naive animals, MTS exposure led to a decrease in the absolute number of all cell types assessed compared with that in sham-GM/OVA-exposed animals (Table I).

Cytokine levels in airway and serum

To determine whether the altered cellular profile in BAL and lung tissue was associated with decreased expression of Th2 effector cytokines and chemokines, we measured levels of IL-5, IL-13, and eotaxin in BAL fluid and IL-13 in the serum of sham- and MTS-GM/OVA-exposed mice. Cytokines were measured 24 h after the ninth OVA aerosolization, the time point at which we have previously demonstrated maximal cytokine expression (30). Interestingly, we observed similar, if not a trend toward enhanced levels of IL-5, IL-13 and eotaxin in the BAL, and IL-13 in the serum of MTS-GM/OVA-exposed mice, compared with sham-GM/OVA-

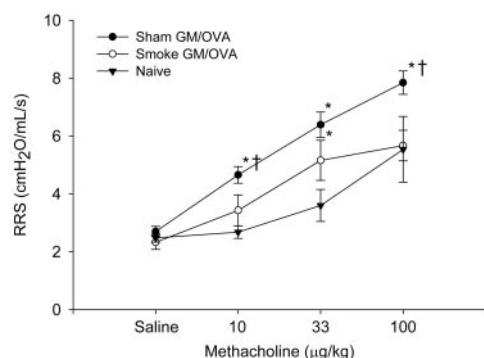


FIGURE 5. Airway hyperresponsiveness to MCh. Mice were either untreated or exposed to room air (sham) or MTS for 2–3 mo and treated with GM/OVA as outlined in *Materials and Methods*. Four weeks later, mice were re-exposed to OVA and airway function was assessed. Data shown represent the mean ± SEM (*n* = 7–9). Statistical analysis was performed using one-way ANOVA with Duncan's post-hoc test; *p* < 0.05 was considered significant. *, Statistically significant compared with naive; †, statistically significant compared with smoke GM/OVA.

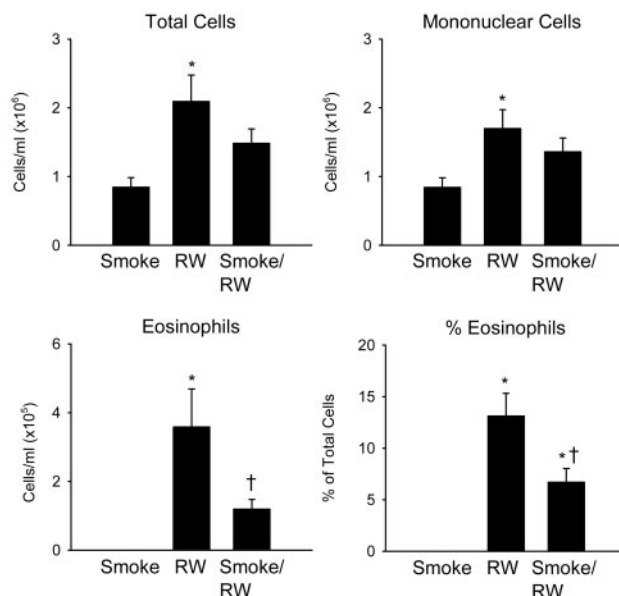


FIGURE 6. Inflammatory profile in the BAL of RW-exposed mice. Mice were exposed to MTS for 5 wk and treated with RW as outlined in *Materials and Methods*. Forty-eight hours after the final RW administration, lungs were lavaged, and the numbers of total cells, mononuclear cells and eosinophils were determined. RW refers to age-matched mice exposed to RW only for 7 days. Data shown represent the mean ± SEM (*n* = 5). Statistical analysis was performed using one-way ANOVA with Fisher's least significant difference post-hoc test; *p* < 0.05 was considered significant. *, Statistically significant compared with smoke alone; †, statistically significant compared with RW only.

exposed animals (Table II). Furthermore, we did not observe Th2 cytokine production in the BAL of naive animals.

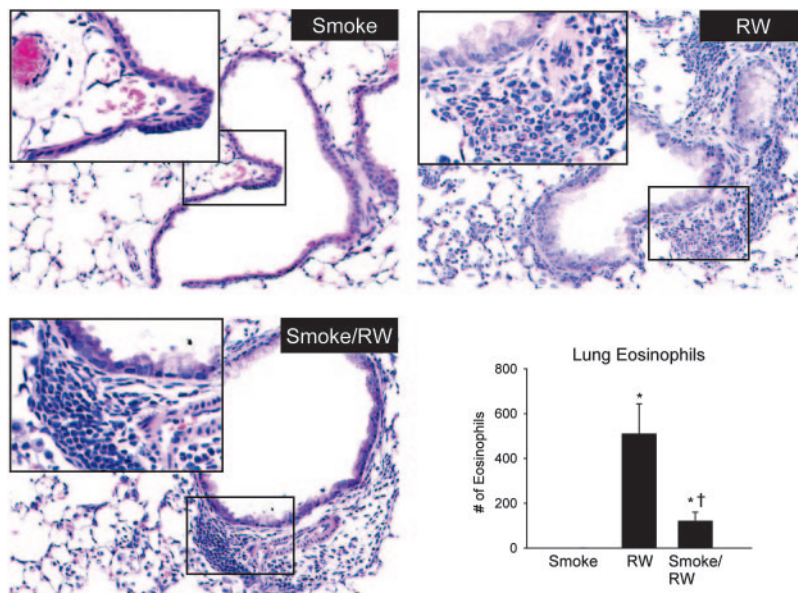
OVA-induced airway hyperresponsiveness

To determine whether the effects of MTS on allergic sensitization and inflammation were associated with physiological changes in the airway, sham- and MTS-GM/OVA-exposed animals were re-challenged with OVA after resolution of the primary immune inflammatory response, and OVA-induced airway hyperresponsiveness to methacholine was assessed. Naive, untreated animals were incorporated to determine control baseline measurements. Sham-GM/OVA animals were significantly more hyperresponsive than naive mice (Fig. 5). Furthermore, MTS-GM/OVA-exposed mice exhibited decreased airway hypersensitivity compared with sham-GM/OVA-exposed animals.

RW-induced sensitization and airways inflammation

To assess whether cigarette smoke impacted allergic responses to allergens other than OVA, we evaluated the impact of MTS on responses to a common environmental aeroallergen, RW. Mice were exposed to cigarette smoke for 5 wk. During the final week of MTS exposure, mice were given RW as outlined in *Materials and Methods*. Mice were killed 48 h after the last RW administration, and the BAL cellular profile was assessed. We observed significantly increased numbers of total cells, mononuclear cells, and eosinophils in animals exposed to RW alone compared with MTS-only-exposed mice (Fig. 6). As in the GM/OVA studies, both the number and the percentage of BAL eosinophils were significantly reduced in MTS-RW-exposed mice compared with RW-only-exposed animals. Furthermore, the decrease in BAL eosinophilia was corroborated by a statistically significant decrease in the number of tissue eosinophils (Fig. 7).

FIGURE 7. Lung tissue eosinophilia in RW-exposed mice. Mice were exposed to MTS for 5 wk and given RW as outlined in *Materials and Methods*. Forty-eight hours after the final RW administration, lungs were isolated, and sections were stained with H&E to visualize eosinophils. RW refers to age-matched mice exposed to RW only for 7 days. Shown are representative histological sections. Data represent the mean $\pm$ SEM ($n = 4-5$). Statistical analysis was performed using one-way ANOVA with Fisher's least significant difference post-hoc test; $p < 0.05$ was considered significant. *, Statistically significant compared with smoke alone; †, statistically significant compared with RW only.



To evaluate the impact of MTS on RW-induced mucus production, another key characteristic of allergic airways disease, lung tissue sections were stained with PAS, and the percentage of positively stained airway perimeter was quantified as outlined in *Materials and Methods*. Fig. 8 demonstrates that animals exposed to RW only and MTS-RW-exposed mice had significantly more PAS airways staining than MTS only-exposed mice. There was also significantly less PAS staining in the airways of MTS-RW-exposed mice compared with animals exposed to RW only.

To assess the impact of MTS on RW-specific systemic sensitization, mice were exposed to MTS for 5 wk. During the final week of MTS exposure, mice were given RW. Forty-eight hours after the last RW administration, mice were killed, and spleen mononuclear cells were isolated and placed in culture in the presence or the absence of RW extract. RW-specific cytokine production was assessed in cell culture supernatants by ELISA. We observed a significant increase in RW-specific levels of IL-4, IL-5, and IL-13 by splenocytes isolated from MTS-RW-exposed animals compared with RW-only-exposed mice (Fig. 9). We observed negligible levels of RW-specific cytokine production by cells isolated

from MTS-only-exposed mice. In addition to cytokines, we evaluated RW-specific IgG1 in the serum of these mice. Fig. 9 shows a significant increase in RW-specific IgG1 production in RW-only-exposed mice, but not in MTS-RW-exposed animals, compared with MTS-only exposure.

Discussion

Although the impact of secondhand or ETS on allergic responses has been described in considerable detail (4, 5, 9–11), the relationship between MTS and allergy remains controversial. The objective of this study was to evaluate the impact of MTS on allergic sensitization and the development of allergic airways inflammation. Specifically, we assessed the effect of MTS on the development of allergic responses toward a surrogate model allergen, chicken egg OVA, and a common environmental aeroallergen, RW.

It is now well established that mucosal exposure to OVA in mice does not lead to allergic inflammation, but instead induces a state of inhalation tolerance (36–38). Therefore, in our studies

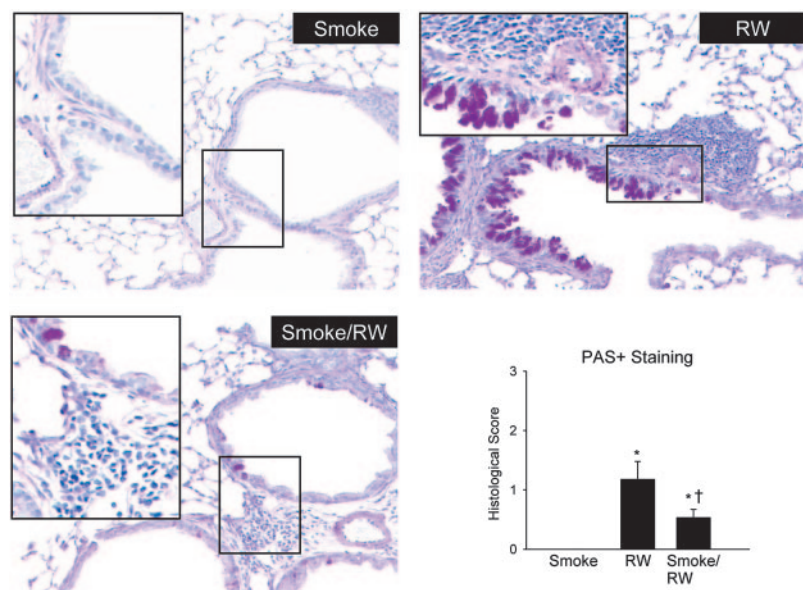


FIGURE 8. Mucus production in the lungs of RW-exposed mice. Mice were exposed to MTS for 5 wk and given RW as outlined in *Materials and Methods*. Forty-eight hours after the final RW administration, lungs were isolated, and sections were stained with PAS to visualize mucus production. RW refers to age-matched mice exposed to RW only for 7 days. Shown are representative histological sections. Data represent the mean $\pm$ SEM ($n = 4-5$). Statistical analysis was performed using one-way ANOVA with Fisher's least significant difference post-hoc test; $p < 0.05$ was considered significant. *, Statistically significant compared with smoke alone; †, statistically significant compared with RW only.

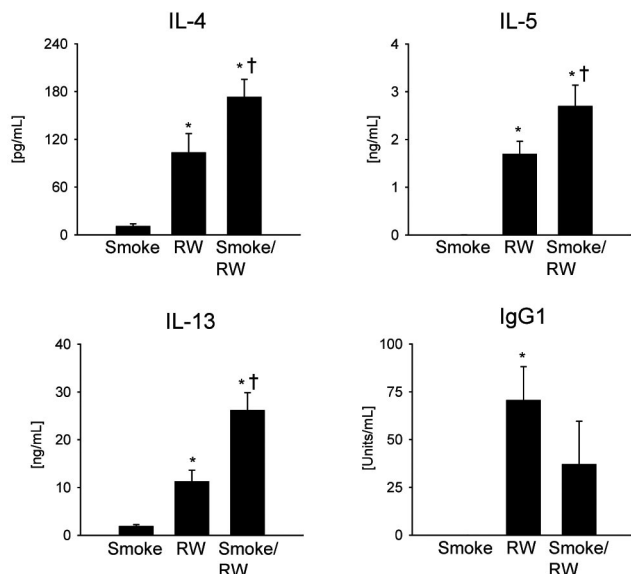


FIGURE 9. In vitro RW-specific cytokine and IgG1 production. Mice were exposed to MTS for 5 wk and given RW as outlined in *Materials and Methods*. Forty-eight hours after the final RW administration, splenocytes were harvested and placed in culture in the presence of either medium alone or RW. RW refers to age-matched mice exposed to RW only for 7 days. Cytokine production was assessed in cell culture supernatants by ELISA. Serum was also collected at the same time point, and RW-specific IgG1 was measured by ELISA. Data are expressed as the mean $\pm$ SEM ($n = 4-5$). Statistical analysis was performed using one-way ANOVA with Fisher's least significant difference post-hoc test; $p < 0.05$ was considered significant. *, Statistically significant from smoke alone; †, statistically significant compared with RW only.

mice were exposed to OVA in the context of GM-CSF, a physiologically relevant adjuvant (39). We have previously shown that GM-CSF expression in the airway prevents tolerance induction and allows for allergic mucosal sensitization to OVA (30). In particular, this experimental approach induces Th2-polarized immune inflammatory responses in the lung including elevated levels of Th2-associated cytokines, Ag-specific Igs, and airway hyperresponsiveness (30, 34). In contrast to what is observed with OVA, recent studies indicate that common environmental allergens such as RW and house dust mite are intrinsically capable of creating an airway milieu that is conducive to allergic sensitization in the absence of exogenous adjuvants (31, 40, 41). For this reason, in a parallel set of studies mice were exposed to MTS and RW.

We report that MTS exposure increased the production of Th2-associated cytokines by splenocytes, suggestive of heightened systemic allergic sensitization. This is probably not the result of Th2 skewing of the immune response, because we observed similar if not increased levels of IFN- γ in sham- and MTS-exposed animals. Furthermore, our observation of similar levels of IL-10 production in sham- and MTS-exposed mice suggests that the increased levels of the Th2 cytokines IL-4, IL-5, and IL-13 was not due to increased production of immune regulatory cytokines. Our data are consistent with human studies that demonstrate increased cytokine production in the peripheral blood of smokers compared with nonsmokers. Specifically, Byron et al. (42) found that IL-4 levels increased in a cigarette dose-dependent manner, and Majori et al. (43) showed a higher percentage of circulating IFN- γ -producing CD4 T lymphocytes in the peripheral blood of smokers compared with nonsmokers.

Despite increased production of Th2 cytokines by splenocytes, MTS attenuated eosinophilic inflammatory responses in both

OVA- and RW-exposed animals. This decrease was observed in BAL and was corroborated by a pronounced reduction in the number of eosinophils in lung tissue (Figs. 4 and 7). This observation is in accordance with a study by Melgert et al. (44), who recently demonstrated that MTS impacts secondary allergic responses. Explicitly, the authors demonstrated reduced airway eosinophilia after Ag challenge when allergic sensitization occurred before the initiation of smoke exposure. Hence, MTS appears to attenuate airways eosinophilia in murine models of experimental asthma, echoing clinical observations of reduced sputum eosinophilia in asthmatic smokers compared with asthmatic nonsmokers (45). These observations may also in part account for reports of decreased asthma risk in smokers (24, 25). Our studies also suggest that increased systemic responsiveness is not necessarily predictive of heightened lung inflammation, cautioning the use of systemic biomarkers to determine the effect of cigarette smoke on lung immune inflammatory responses.

MTS exposure did not impact the production of OVA-specific IgE and IgG1, Igs commonly associated with Th2 responses. Interestingly, OVA-specific IgG2a production was attenuated by ~75% in MTS- compared with sham-exposed mice. Previous reports have shown that cigarette smoking decreases serum levels of most Ig classes, except for IgE (46–51). For example, lower levels of precipitating Abs have been reported in smoking pigeon breeders (47) and smokers working in poultry plants (48), cotton mills (49), and cigar plants (50). Furthermore, we have previously demonstrated that MTS decreased Ig responses after adenoviral infection (32), and we are actively pursuing studies in our laboratory to determine whether the altered Ig profile associated with MTS exposure is a consequence of smoke-induced deregulation of T cell function and/or is a direct effect of cigarette smoke on either B cell function or clearance of Ab from the blood.

We have previously shown that OVA exposure in the absence of an appropriate adjuvant (i.e., GM-CSF or aluminum hydroxide) induces inhalation tolerance (30). Interestingly, reports that children exposed to ETS are at increased risk to develop childhood asthma (4, 5) together with studies in mice reporting that ETS, in itself, is capable of both inducing and exacerbating allergic responses to OVA (9–11) collectively suggest that ETS may surmount the innocuous nature of OVA, leading to allergic airways inflammation. In contrast, we show that MTS did not facilitate the development of OVA-induced airways inflammation (Fig. 3). Therefore, our results suggest that there are marked differences in the biological outcomes attributed to exposure to either MTS or ETS. Specifically, in the context of allergen exposure, MTS and ETS may demonstrate anti- and proinflammatory properties, respectively, and could reflect variation in the composition and concentration of constituents found in both types of tobacco smoke (52).

Allergen-specific CD4 Th responses are indispensable to the development and maintenance of allergic inflammatory processes (53, 54). In this study, MTS exposure resulted in fewer activated (CD69⁺) and Th2-associated (T1ST2⁺) CD4 T cells after GM/OVA exposure as well as reduced the number of B cells and dendritic cells in the lung. This effect appeared to be specific to the lung tissue because cigarette smoke did not affect the number of mononuclear cells in the BAL. Interestingly, we observed similar levels of IL-5, IL-13, and eotaxin in sham- and smoke-exposed animals, suggesting that in the context of MTS exposure, fewer cells were capable of producing equivalent levels of cytokines as in sham exposure. This observation was consistent with the enhanced capacity of MTS-exposed animals to produce OVA- and RW-specific cytokines in vitro and suggests that the inhibitory effect of MTS on immune inflammatory responses in the lung is not attributed to attenuation of allergen-specific sensitization.

The accumulation of eosinophils in the airways is multifaceted, involving eosinopoiesis, migration, and diapedesis. A number of cytokines, chemotactic factors, adhesion molecules, and proteolytic enzymes facilitate these processes. That we observed similar levels of IL-5 in the BAL and equivalent numbers of eosinophils in the peripheral blood of both sham- and MTS-GM/OVA-exposed mice (data not shown) decreases the likelihood that MTS exposure impaired eosinopoiesis. We also show that eotaxin levels were similar between the experimental groups, demonstrating that cigarette smoke did not inhibit the production of an important eosinophil chemotactic factor. It remains to be determined whether MTS affects the production of other chemokines, cell surface expression of their receptors, and/or intercellular adhesion molecules. Alternatively, it may also be argued that cigarette smoke is toxic to immune inflammatory cells and that the reduced number of eosinophils in the tissue and BAL is a reflection of this toxicity. For example, it has recently been shown that activated eosinophils are susceptible to the noxious effects of NO, a component of cigarette smoke (55).

Although poorly understood, the mechanism underlying the development of airways hyperresponsiveness, a key clinical feature of allergic disease, has been associated with increased inflammation in the airway, augmented Th2 cytokine production, and elevated circulating levels of IgE. Because MTS exhibited dissimilar effects on sensitization and the ensuing immune inflammatory response, it was difficult to predict an effect for MTS on allergen-induced physiological changes in the airway. The finding that cigarette smoke significantly attenuated airway hypersensitivity and maximal bronchoconstriction (Fig. 5) may therefore be a result of reduced inflammation and/or mucus production (as seen in RW-sensitized animals) in the lungs of these animals. Alternatively, MTS may have inflammation-independent effects on airway smooth muscle cell function.

Cigarette smoke contains appreciable levels of bacterial endotoxin (56), and there is a growing body of literature demonstrating that LPS has the capacity to modulate allergic responses (57–60). Hence, it could be argued that the attenuated airway inflammatory response observed in MTS-exposed animals is due in part to endotoxin contained within cigarette smoke. However, commercial preparations of OVA have also been shown to contain bacterial endotoxins that may attenuate or exacerbate allergic responses depending on the experimental conditions (57–60). Although Eisenbarth et al. (58) have shown that low doses of endotoxin exacerbate the inflammatory response to OVA, Watanabe et al. (57) showed that contaminating endotoxin in commercial OVA preparations significantly attenuated endothelial cell activation, airway hyperresponsiveness, and lung inflammatory responses. This effect was attributed to the generation of endotoxin tolerance induced by sensitization with endotoxin before allergen exposure. Hence, our experimental system is probably too complex to assess whether attenuated airway inflammation was due to LPS exposure (56). Although the biological significance of endotoxin contained in cigarette smoke is of interest, tobacco smoke contains >4500 components. By exposing mice to MTS, we assess the effects of all these compounds collectively on the immune system. Ultimately, whether the effects we observed are due to a single entity, such as LPS, or the combined effects of multiple agents remains to be addressed.

That cigarette smoke attenuated certain aspects of the allergic response is in keeping with previous reports by us and others that MTS demonstrates immunosuppressive properties (32; reviewed in Ref. 61). In certain contexts then, specific components within MTS may be of therapeutic benefit in the treatment of immune-driven disorders. Indeed, recent studies have shown that nicotine

exposure diminished the development of experimental granulomatous lung disease (62) and ulcerative colitis (63) in mice. In contrast, these same immunosuppressive properties may be detrimental to the host. It has recently been demonstrated in mice, for example, that although in vivo nicotine exposure inhibited influenza-mediated inflammatory responses in the lung, viral burden was significantly increased compared with that after sham treatment (64). Furthermore, we have shown that in addition to delaying pulmonary clearance of *Pseudomonas aeruginosa* in mice (65), MTS exposure decreased serum levels of virus-specific IgG and diminished viral neutralization capacity after adenovirus infection (32). The fact that MTS suppresses both cellular and humoral local immune responses may consequently impact the ability to adequately control pulmonary infections, a common etiologic factor in the development of clinical exacerbation of asthma (66, 67).

In summary, we demonstrate that MTS exposure was associated with increased cytokine production by splenocytes in response to both OVA and RW challenge. Paradoxically, MTS exposure resulted in an overall attenuation of the immune inflammatory response in the lung and diminished airway hyperresponsiveness. MTS, therefore, plays a disparate role in the development of allergic responses, inducing a heightened state of allergen-specific sensitization, but dampening local immune inflammatory processes.

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Disclosures

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