

Thomas H. Thatcher, Randi P. Benson, Richard P. Phipps and Patricia J. Sime
Am J Physiol Lung Cell Mol Physiol 295:412-421, 2008. First published Jun 20, 2008;
doi:10.1152/ajplung.00392.2007

You might find this additional information useful...

This article cites 53 articles, 23 of which you can access free at:

<http://ajplung.physiology.org/cgi/content/full/295/3/L412#BIBL>

Updated information and services including high-resolution figures, can be found at:

<http://ajplung.physiology.org/cgi/content/full/295/3/L412>

Additional material and information about *AJP - Lung Cellular and Molecular Physiology* can be found at:

<http://www.the-aps.org/publications/ajplung>

This information is current as of November 6, 2008 .

High-dose but not low-dose mainstream cigarette smoke suppresses allergic airway inflammation by inhibiting T cell function

Thomas H. Thatcher,¹ Randi P. Benson,² Richard P. Phipps,² and Patricia J. Sime^{1,2}

¹Department of Medicine, and ²Department of Environmental Medicine and the Lung Biology and Disease Program, University of Rochester, Rochester, New York

Submitted 19 September 2007; accepted in final form 19 June 2008

Thatcher TH, Benson RP, Phipps RP, Sime PJ. High-dose but not low-dose mainstream cigarette smoke suppresses allergic airway inflammation by inhibiting T cell function. *Am J Physiol Lung Cell Mol Physiol* 295: L412–L421, 2008. First published June 20, 2008; doi:10.1152/ajplung.00392.2007.—Epidemiological studies have identified childhood exposure to environmental tobacco smoke as a significant risk factor for the onset and exacerbation of asthma, but studies of smoking in adults are less conclusive, and mainstream cigarette smoke (MCS) has been reported to both enhance and attenuate allergic airway inflammation in animal models. We sensitized mice to ovalbumin (OVA) and exposed them to MCS in a well-characterized exposure system. Exposure to MCS (600 mg/m³ total suspended particulates, TSP) for 1 h/day suppresses the allergic airway response, with reductions in eosinophilia, tissue inflammation, goblet cell metaplasia, IL-4 and IL-5 in bronchoalveolar lavage (BAL) fluid, and OVA-specific antibodies. Suppression is associated with a loss of antigen-specific proliferation and cytokine production by T cells. However, exposure to a lower dose of MCS (77 mg/m³ TSP) had no effect on the number of BAL eosinophils or OVA-specific antibodies. This is the first report to demonstrate, using identical smoking methodologies, that MCS inhibits immune responses in a dose-dependent manner and may explain the observation that, although smoking provokes a systemic inflammatory response, it also inhibits T cell-mediated responses involved in a number of diseases.

cigarette smoking; asthma; allergy; T helper 2; T cells

CIGARETTE SMOKING IS WIDELY recognized as a cause or significant risk factor for a number of diseases, including chronic obstructive pulmonary disease, lung cancer, and cardiovascular disease (4, 7, 21, 25, 39). Recent research has focused on the role of systemic chronic inflammation in the pathogenesis of these conditions (24, 52), and cigarette smoke is a strong proinflammatory stimulus (26, 44, 45, 53). Indeed, cigarette smoking is also a risk factor for systemic diseases with inflammatory components such as diabetes and atherosclerosis (11, 24). It could be reasonably expected that cigarette smoking will have an adverse effect on any disease in which chronic inflammation plays a significant role. However, there is evidence that cigarette smoking may decrease the incidence or severity of some chronic inflammatory diseases including sarcoidosis and pigeon fanciers' lung (2, 3, 41). Additionally, smokers have increased susceptibility toward bacterial and viral infections, suggesting that, although smoking promotes inflammation, it may also inhibit bacterial and viral clearance (2, 31).

The relationship between cigarette smoking and asthma is complex and poorly understood. Epidemiological studies have

identified childhood exposure to environmental tobacco smoke (ETS, or "second-hand smoke") as a significant risk factor for the onset and exacerbation of asthma (8, 13, 14, 32). In adults, some studies have shown an association between both active and passive smoking and asthma severity (8–10), whereas other studies have failed to find such associations (38, 49). It has also been reported that cigarette smoking can attenuate allergic asthma and rhinoconjunctivitis in a dose-dependent manner (17) and that, whereas smokers generally have higher levels of circulating IgE antibodies, they are less likely than nonsmokers to be sensitized to some environmental allergens (18, 19).

Attempts to model the effects of cigarette smoking on asthma in animals have yielded contradictory results. Low levels of ETS enhance the development of allergic airway inflammation (35). However, mainstream cigarette smoke (MCS) has been reported to both attenuate (28, 34) and enhance (29, 30, 36) allergic airway inflammation. Unfortunately, direct comparison of these reports is complicated by the fact that they used different strains of mice, different routes of allergic sensitization, and different methods of MCS exposure.

To further explore the role of MCS exposure on allergic airway inflammation, we sensitized mice to the model allergen ovalbumin (OVA) and then exposed them to MCS both before and after challenge with inhaled OVA aerosol. We report here for the first time that exposure to MCS significantly inhibits allergic airway inflammation by inhibiting T helper proliferation and cytokine production. We also report that lower levels of MCS exposure have no effect on the development of airway inflammation and OVA-specific antibodies. We conclude that MCS suppresses allergic responses in a dose-dependent manner, which may explain the previously inconsistent reports on the effects of smoking on asthma.

MATERIALS AND METHODS

Cigarette smoke and OVA exposures. All animal procedures were approved and supervised by the University Committee on Animal Research. Adult female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8–12 wk of age. OVA aerosol exposures were performed by placing the mice in individual compartments of a wire cage, which was placed inside a closed plastic box connected to a nebulizer (Salter, Arvin, CA) containing OVA (5 mg/ml in normal saline; Sigma, St. Louis, MO) and connected to medical grade compressed air at 6 psi. Control animals were exposed to saline aerosol in an identical apparatus.

Cigarette smoke exposures were performed by placing the mice in individual compartments of a wire cage, which was placed inside a

Address for reprint requests and other correspondence: P. J. Sime, Univ. of Rochester, Division of Pulmonary and Critical Care Medicine, 601 Elmwood Ave., Box 692, Rochester, NY 14642 (e-mail: patricia_sime@urmc.rochester.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

closed plastic box connected to a Baumgartner-Jaeger CSM2072i cigarette smoking machine (CH Technologies, Westwood, NJ). Research cigarettes (1R3F, University of Kentucky) were smoked according to the FTC protocol (1 puff/min per cigarette of 2-s duration and 35-ml volume). MCS was diluted with filtered air and directed into the exposure chamber. Smoke exposures were quantified by determining total suspended particulate matter (TSP) with a Microdust Pro aerosol monitor (Casella CEL, Bedford, United Kingdom) and by daily gravimetric sampling. The smoke concentration was set at a nominal value of 600 or 80 mg/m³ TSP by adjusting the number of cigarettes loaded onto the carousel and the flow rate of the dilution air. The average actual exposure for these experiments was 588 ± 52 mg/m³ TSP for the high-dose exposure and 77 ± 10 mg/m³ TSP for the low dose. Control animals were exposed to filtered air in identical equipment on the same schedule (45). (Separate cages and boxes were used for smoke, air, OVA, and saline.)

Experimental design. Four groups of mice were established: Saline/Air (control), Saline/MCS, OVA/Air, and OVA/MCS. Mice were sensitized to OVA with a single intraperitoneal injection of 8 µg of OVA with 4 mg of aluminum hydroxide (Sigma) in 0.5 ml of normal saline, or saline alone, on day 0, followed by a single 1-h exposure to OVA or saline aerosol on day 5. Beginning on day 6, mice were exposed to MCS for 1 h/day, 6 days/wk. On day 19, all mice were challenged with 2 1-h exposures to OVA aerosol. Mice were killed on days 20, 22, and 26 (1, 3, and 7 days postchallenge) with MCS exposures continuing daily until death (Fig. 1).

Tissue harvest and bronchoalveolar lavage. Mice were anesthetized with 2,2,2-tribromoethanol (Avertin, 250 mg/kg ip) and killed by exsanguination. Blood was collected by cardiac puncture, and the serum fraction was stored for later analysis. The heart and lungs were removed en bloc, and the lungs were lavaged twice with 0.5 ml of PBS. The lavage fluid was centrifuged, and the cell-free supernatants were frozen for later analysis. The bronchoalveolar lavage (BAL) cell pellet was resuspended in PBS, and the total cell number was determined by counting on a hemacytometer. Differential cell counts (minimum of 300 cells per slide) were performed on cytospin-prepared slides (Thermo Shandon, Pittsburgh, PA) stained with Diff-Quik (Dade Behring, Newark, DE). The lungs were frozen in liquid nitrogen for later analysis. Some lungs were inflated and fixed in 10% neutral buffered formalin without undergoing lavage. Tissues were embedded in paraffin, sectioned (5 µm), and stained with hematoxylin and eosin (H&E) or with periodic acid-Schiff reagent (PAS).

Cytokine analysis. One lobe of the lung was homogenized in 1 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, and 1 mM DTT containing protease inhibitors; Sigma). Nonidet P-40 was added to 0.5%, and the homogenates were vortexed and left on ice for 15 min. Supernatants were collected after centrifugation at 10,000 g for 10 min. Protein concentrations were measured by bicinchoninic acid (BCA) colorimetric assay (Pierce, Rockford, IL). IL-4 and IL-5

were measured in homogenates by cytokine multiplex analysis (Beadlyte; Millipore, Temecula, CA) and read on a Luminex 100 (Luminex, Austin, TX) and expressed as picograms of cytokine per milligram of total protein in the homogenate. PGE₂ was measured in BAL fluid (BALF) as previously described (22). The limit of detection was 15 pg/ml.

Eosinophil peroxidase assay. Frozen lung tissue was homogenized in water containing protease inhibitors (Sigma) and centrifuged for 10 min at 16,000 g. The supernatant was decanted, and the pellet rehomogenized in water containing protease inhibitors and 0.5% cetyltrimethylammonium chloride (CTAC; Sigma). The homogenate was subjected to 1 freeze-thaw cycle, sonicated with a probe sonicator (Branson Ultrasonics, Danbury, CT), and then centrifuged for 10 min at 10,000 g. Eosinophil peroxidase (EPO) activity was measured in the supernatant by mixing 50 µl of the supernatant with 1 ml of assay buffer [50 mM HEPES, pH 6.5, 6 mM potassium bromide, 1.5 mM *o*-phenylenediamine dihydrochloride (OPD; Sigma), and 4.4 mM hydrogen peroxide] and monitoring the reaction kinetically at 425 nm (47). Enzyme activity was calculated using the molar extinction coefficient of OPD at 425 nm, $E = 5,300 \text{ M}^{-1}\text{cm}^{-1}$ (1). One unit of peroxidase activity degrades 1 µmol of substrate per minute. Total protein in the lung homogenate was measured by BCA assay, and peroxidase activity was reported as units of activity per milligram of protein in the homogenate. EPO activity was measured in BALF by mixing 0.2 ml of BALF with 0.2 ml of 2× assay buffer.

Lymph node cell proliferation and cytokine assays. At death, the peribronchial and mediastinal lymph nodes were removed, and lymph node cells were harvested by crushing the nodes between sterile frosted glass slides (6 mice per group). Because the lymph nodes from nonsensitized mice (Saline/Air and Saline/MCS groups) were very small, nodes from 3 mice were pooled for culturing. For the cell proliferation assay, 1×10^5 cells were plated per well of a 96-well plate and cultured in HL-1 serum-free medium (Lonza, Walkersville, MD) as previously described (46). Cells were cultured for 4 days with 50 µg/ml OVA, with 1 µCi [methyl-³H]thymidine (DuPont/NEN Research Products, Boston, MA) added for the last 18 h. Cells were harvested onto glass fiber filters using a Packard MicroMate 196 cell harvester (Packard, Meriden, CT), and ³H incorporation was counted with a Packard Matrix 96 Direct Beta Counter. Background proliferation was measured in wells without added antigen. Con A (Sigma) was added to some wells at a final concentration of 1.5 µg/ml as a positive control. Cultures were performed in duplicate, and the results shown are the average of 6 mice (or 2 pools of 3 mice each) per group after subtraction of background counts.

To measure cytokine production, 4×10^5 lymph node cells were plated per well of a 96-well plate and cultured with 50 µg/ml OVA, 1.5 µg/ml Con A, or media alone. After 72 h, the supernatants were harvested and assayed for IL-4 and IFN-γ by commercial ELISA (R&D Systems).

Immunoglobulin measurements. Total immunoglobulin in serum (IgG₁, IgG_{2a}, and IgE) was measured by sandwich ELISA using a purified monoclonal antibody as the capture antibody and a biotinylated monoclonal antibody (a different clone) as the detection antibody (BD Biosciences, San Jose, CA). Bound Ig was detected with alkaline phosphatase-conjugated streptavidin and alkaline phosphatase substrate kit (Bio-Rad, Hercules, CA) and compared with a standard curve consisting of purified Ig isotype (BD Biosciences).

For determination of OVA-specific antibodies, ELISA plates (Immulon 4 HBX; Thermo Electron, Milford, MA) were coated with 10 µg/ml OVA in PBS overnight, blocked with 0.5% BSA in PBS, and incubated with diluted serum. Detection was with isotype-specific biotinylated antibody as described above. A laboratory standard was made by pooling sera from 6 mice immunized with OVA in aluminum hydroxide (twice, 5 days apart). This standard was carefully titrated to determine the lowest dilution at which specific binding activity (a signal greater than 3 SD over background) was detected. This dilution was defined as 1 titer unit/ml. A single pooled serum standard

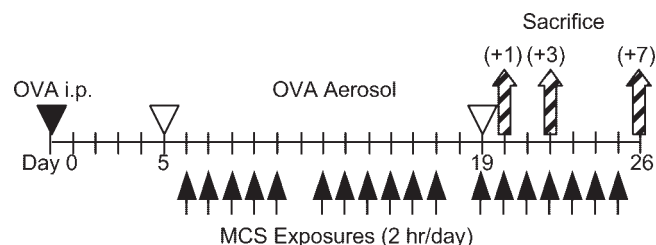


Fig. 1. Ovalbumin (OVA) sensitization and smoke exposure experimental design. BALB/c mice were sensitized to OVA via a single intraperitoneal injection with OVA plus aluminum hydroxide (closed triangle) and 2 aerosol OVA challenges (open triangles). Mice were exposed to air or cigarette smoke for 1 h/day, 6 days/wk, beginning after the 1st OVA aerosol exposure and daily following OVA challenge (black arrows). The mice were killed for analysis of airway inflammation 1, 3, or 7 days after the 2nd OVA challenge (striped arrows). MCS, mainstream cigarette smoke.

containing 30,000 U/ml OVA-specific IgE, 50,000 U/ml OVA-specific IgG_{2a}, and 1×10^6 U/ml OVA-specific IgG₁ was frozen in small aliquots and used throughout the study.

Statistical analysis. All results are reported as the means $\pm$ SE. Statistical significance was assessed by one-factor ANOVA and Student's *t*-test. Values that were not normally distributed were analyzed by the Mann-Whitney nonparametric test. A *P* value <0.05 was considered significant.

RESULTS

MCS exposure inhibits allergic airways eosinophilia. To investigate whether cigarette smoke alters allergic airway inflammation, groups of BALB/c mice were sensitized to the model allergen OVA and then exposed to MCS 6 days/wk for

2 wk before allergen challenge (Fig. 1). The mice were harvested 1, 3, and 7 days following a challenge with inhaled OVA aerosol or 3 days following a control saline exposure. Mice exposed to MCS only exhibited a significant increase in total BAL cells, consisting largely of macrophages and neutrophils (Fig. 2). This is consistent with results we (45) previously obtained with this smoking protocol in C57BL/6 mice. Mice exposed to OVA alone developed significant eosinophilia, a hallmark of allergic inflammation, as well as increased numbers of lymphocytes and macrophages. In contrast, the BALF from OVA/MCS mice contained elevated numbers of macrophages and neutrophils but dramatically reduced numbers of eosinophils. Although the increase in neutrophils was

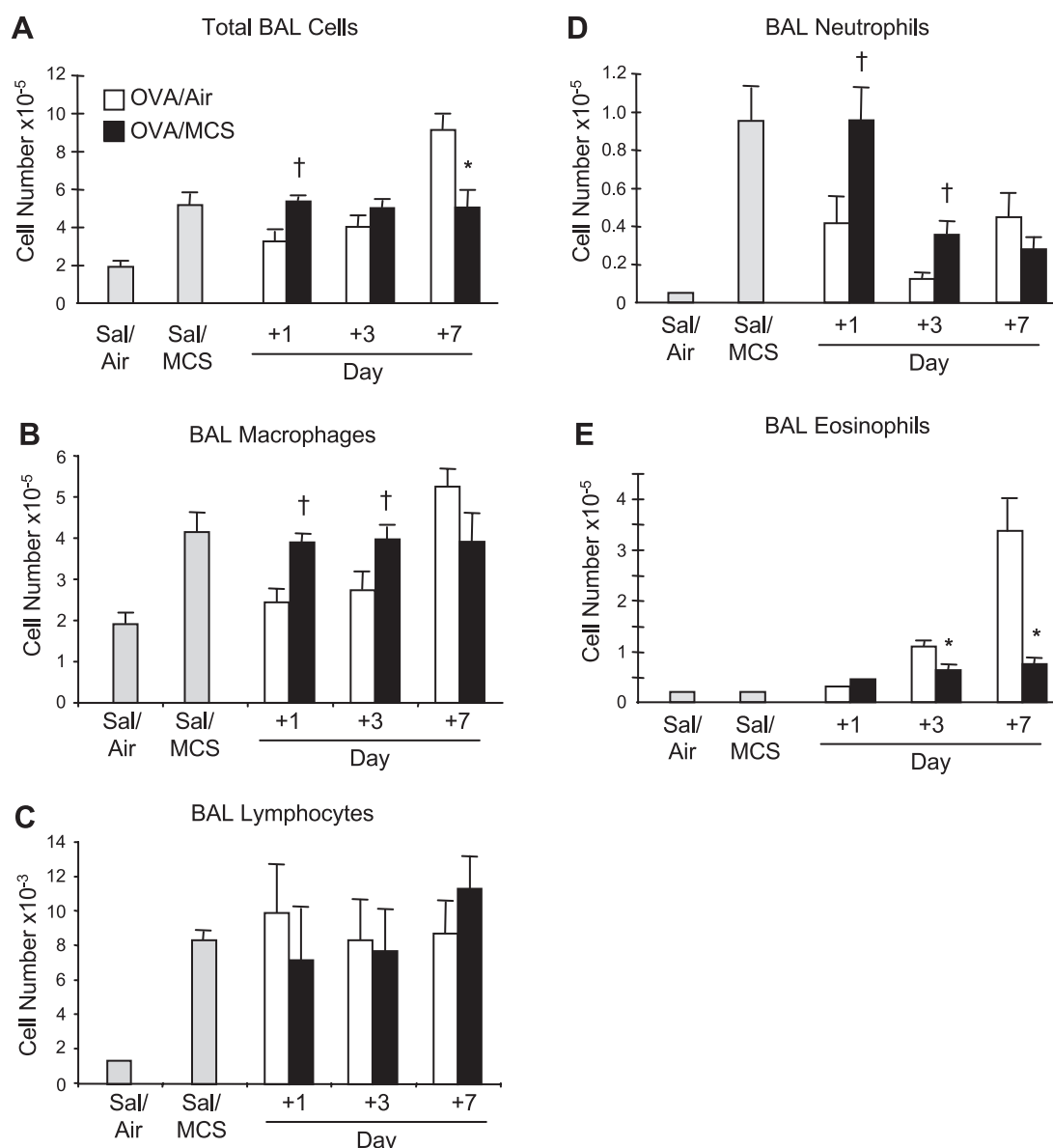


Fig. 2. Cigarette smoke exposure inhibits airway eosinophilia in OVA-sensitized mice. BALB/c mice were exposed to air (white bars) or MCS (black bars) after sensitization with OVA. After 21 days, the mice were all challenged with an OVA aerosol and killed 1, 3, or 7 days after challenge. Differential counts were performed on bronchoalveolar lavage (BAL) cells, and the total cell number (A), number of macrophages (B), lymphocytes (C), neutrophils (D), and eosinophils (E) are reported. Saline/Air (Sal/Air) and Saline/MCS control groups were harvested at day 3 (gray bars). Results shown are the means $\pm$ SE for $n = 5-6$ mice per group and are representative of 3 independent experiments. †Significant increase in OVA-sensitized/MCS-exposed mice compared with OVA-sensitized/air-exposed; *significant decrease in OVA-sensitized/MCS-exposed mice compared with OVA-sensitized/air-exposed ($P < 0.05$, *t*-test and ANOVA).

transient, the reduction in eosinophils was prolonged. Peak eosinophil numbers were reduced 78% on day 7 in MCS-exposed compared with air-exposed mice.

MCS inhibits lung inflammation and goblet cell hyperplasia. The MCS exposure protocol used in this study elicits a mild acute inflammation marked by increased alveolar macrophages and perivascular accumulation of inflammatory cells (Fig. 3*B*). Mice sensitized and challenged with OVA also develop perivascular infiltrates, characterized by frequent eosinophils (Fig. 3, *C* and *E*). However, OVA-challenged mice exposed to MCS developed greatly reduced tissue inflammation, in which eosinophils were sparsely observed (Fig. 3, *D* and *F*). The observation of reduced eosinophilia was confirmed biochemically by measuring EPO activity. Whereas MCS alone had no effect on basal EPO activity

in lung tissue, OVA challenge resulted in a sixfold increase in EPO activity. MCS exposure of OVA-challenged mice resulted in a 65% inhibition of EPO activity (Fig. 4*A*). The effect was equally dramatic in BALF, where MCS inhibited 75% of the increase in EPO activity following OVA challenge (Fig. 4*B*).

Another important hallmark of allergic airways inflammation is increased mucus production, detected by PAS staining for goblet cells. In mice, goblet cells are generally absent from bronchioles and smaller conducting airways (16). The allergic response to OVA provokes goblet cell hyperplasia and mucus production, with nearly all of the epithelial cells of the small conducting airways exhibiting positive PAS staining (Fig. 3*G*). Comparison of airways of similar caliber revealed dramatically reduced PAS staining OVA-challenged mice exposed to MCS (Fig. 3*H*).

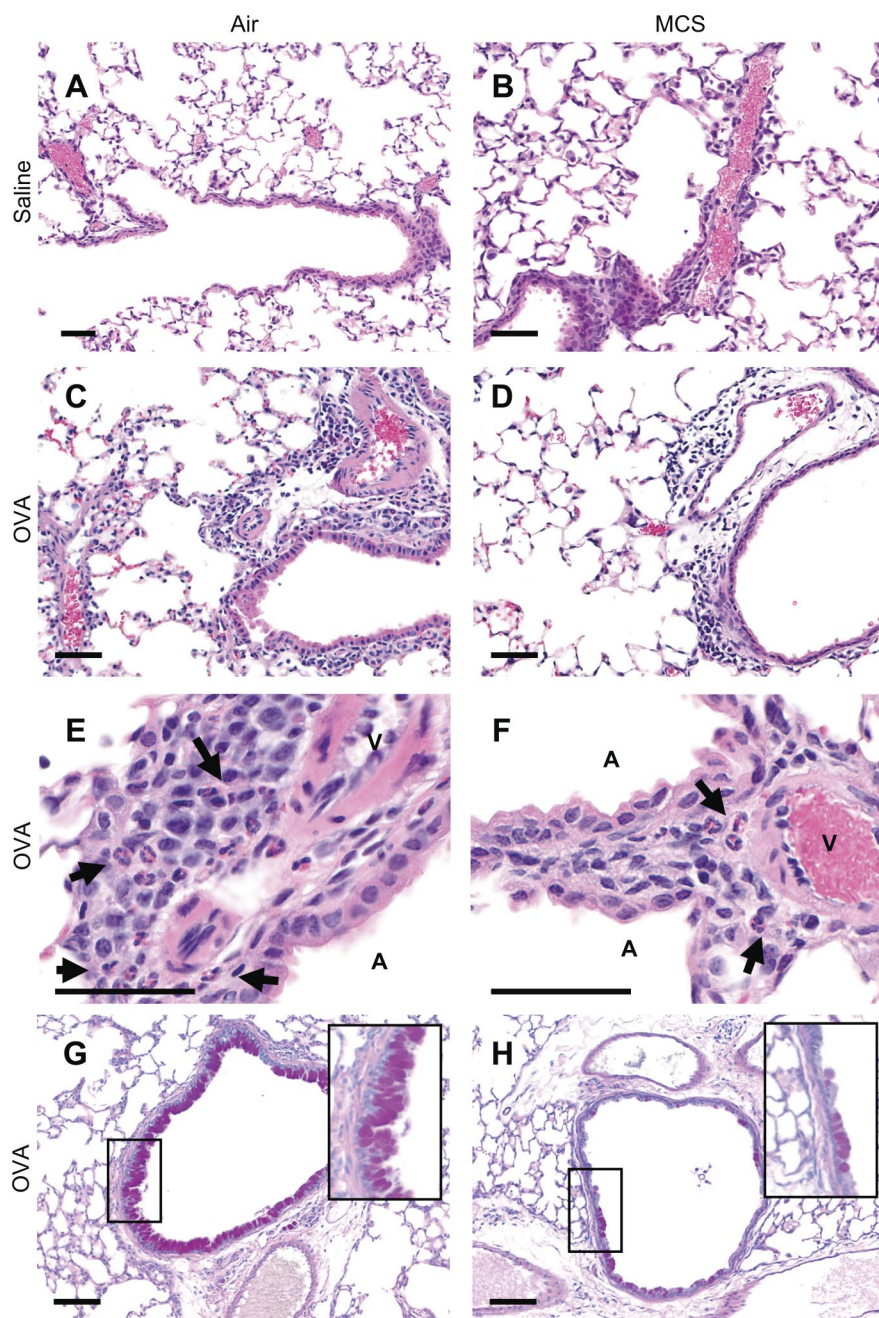


Fig. 3. Cigarette smoke exposure inhibits lung inflammation in OVA-sensitized mice. Mice were sensitized to OVA or saline and exposed to air or smoke as described and killed 7 days after the final OVA aerosol challenge. Lungs were inflated and fixed with formalin, and sections were stained with hematoxylin and eosin (H&E). *A*: air-exposed mice have normal alveoli and blood vessels. *B*: sham-immunized mice exposed to cigarette smoke develop mild inflammation with perivascular infiltrates of lymphocytes and neutrophils and increased numbers of alveolar macrophages. *C*: OVA-sensitized mice develop extensive lymphocytic inflammation around airways and blood vessels after OVA challenge. *D*: OVA-sensitized mice exposed to cigarette smoke develop attenuated inflammation, with fewer and smaller peribronchial and perivascular infiltrates. *E* and *F*: high-magnification views showing that perivascular inflammation in smoke-exposed mice contain fewer eosinophils. A, airway lumen; V, vascular lumen; arrows, eosinophils. *G* and *H*: periodic acid-Schiff (PAS) stain reveals a dramatic decrease in PAS-stained cells in the airways of OVA-sensitized mice exposed to cigarette smoke. Bar = 50 μ m.

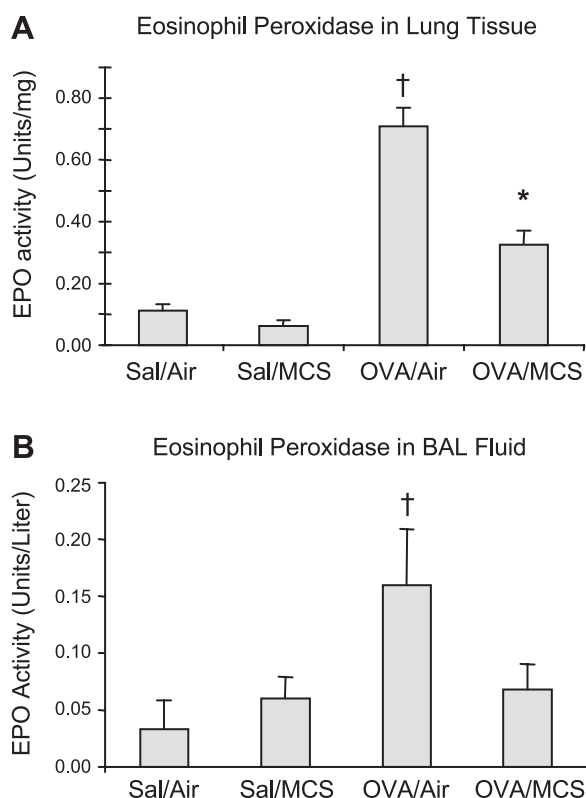


Fig. 4. Cigarette smoke exposure reduces eosinophil peroxidase (EPO) activity in lung tissue and BAL fluid. Mice were exposed to air or MCS after sensitization with OVA as described. After 21 days, the mice were challenged with OVA aerosol and killed 7 days after challenge. EPO activity was measured in lung tissue homogenates (A) or BAL fluid (B). Results shown are the means $\pm$ SE for $n = 5-6$ mice per group. [†]Significant increase in OVA-sensitized/air-exposed mice compared with sham-sensitized (saline) mice; *significant decrease in OVA-sensitized/smoke-exposed mice compared with OVA-sensitized/air-exposed mice ($P < 0.05$, ANOVA).

MCS exposure attenuates lung cytokine levels. The allergic response to OVA includes transient expression of the T helper 2 (Th2) cytokine IL-4 and the eosinophilic cytokine IL-5 in the lungs. MCS exposure resulted in significant reductions in levels of IL-4 and IL-5 in both BALF (data not shown) and lung homogenates from OVA-challenged mice (Fig. 5, A and B). We also examined levels of PGE₂, which promotes Th2 differentiation and is upregulated in human asthma patients (23, 40). Whereas MCS alone did not upregulate PGE₂ levels, OVA challenge resulted in a dramatic and prolonged increase in BAL PGE₂ that was not altered by MCS exposure (Fig. 5C).

MCS exposure inhibits antibody production. OVA sensitized and challenged BALB/c mice have increased levels of total serum IgE and develop high-titer OVA-specific IgE antibodies. MCS exposure had only a modest effect on total IgE levels, with a significant reduction seen only 7 days after challenge (Fig. 6A). MCS also delayed the development of high-titer OVA-specific IgE antibodies (Fig. 6B) and significantly suppressed development of high-titer OVA-specific IgG₁ (Fig. 6C). Whereas IgE and IgG₁ are associated with a Th2 immune response, production of IgG_{2a} is associated with a Th1 response (43). MCS also inhibited the development of OVA-specific IgG_{2a} antibodies, suggesting that MCS does not inhibit the formation of a Th2 response, but rather may be broadly immunosuppressive.

MCS exposure inhibits T cell function. To more closely examine the effect of MCS on the allergic response to OVA, lymph node cells were harvested from the peribronchial lymph nodes of saline-treated and OVA-sensitized and challenged mice. After stimulation in vitro with OVA, production of IL-4

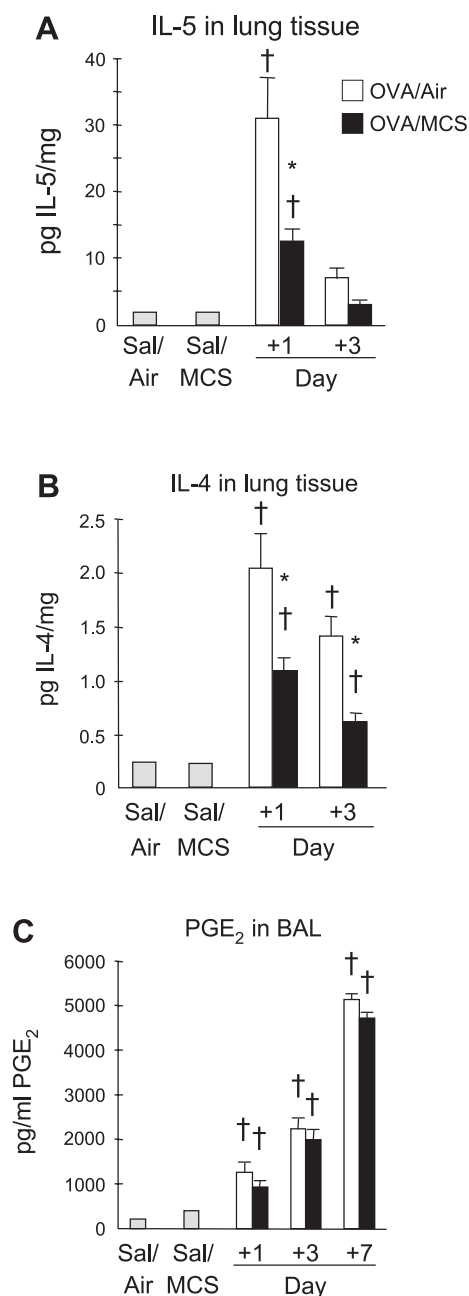


Fig. 5. Cigarette smoke exposure attenuates levels of allergic and inflammatory cytokines. Mice were exposed to air (white bars) or MCS (black bars) after sensitization with OVA or saline and harvested 1, 3, or 7 days after OVA challenge, as described. Saline/Air and Saline/MCS control groups were harvested at day 3 (gray bars). IL-5 (A) and IL-4 (B) were measured in lung homogenates by cytokine multiplex assay as described. PGE₂ (C) was measured in BAL fluid by ELISA. The limit of detection was 0.2 pg/mg for IL-4 and IL-5 and 15 pg/ml for PGE₂. Results are representative of 3 independent experiments. *Significant decrease in OVA-sensitized/smoke-exposed mice compared with OVA-sensitized/air-exposed mice ($P < 0.05$). [†]Significant increase in OVA/Air and OVA/MCS mice compared with Sal/Air and Sal/MCS ($P < 0.01$, ANOVA).

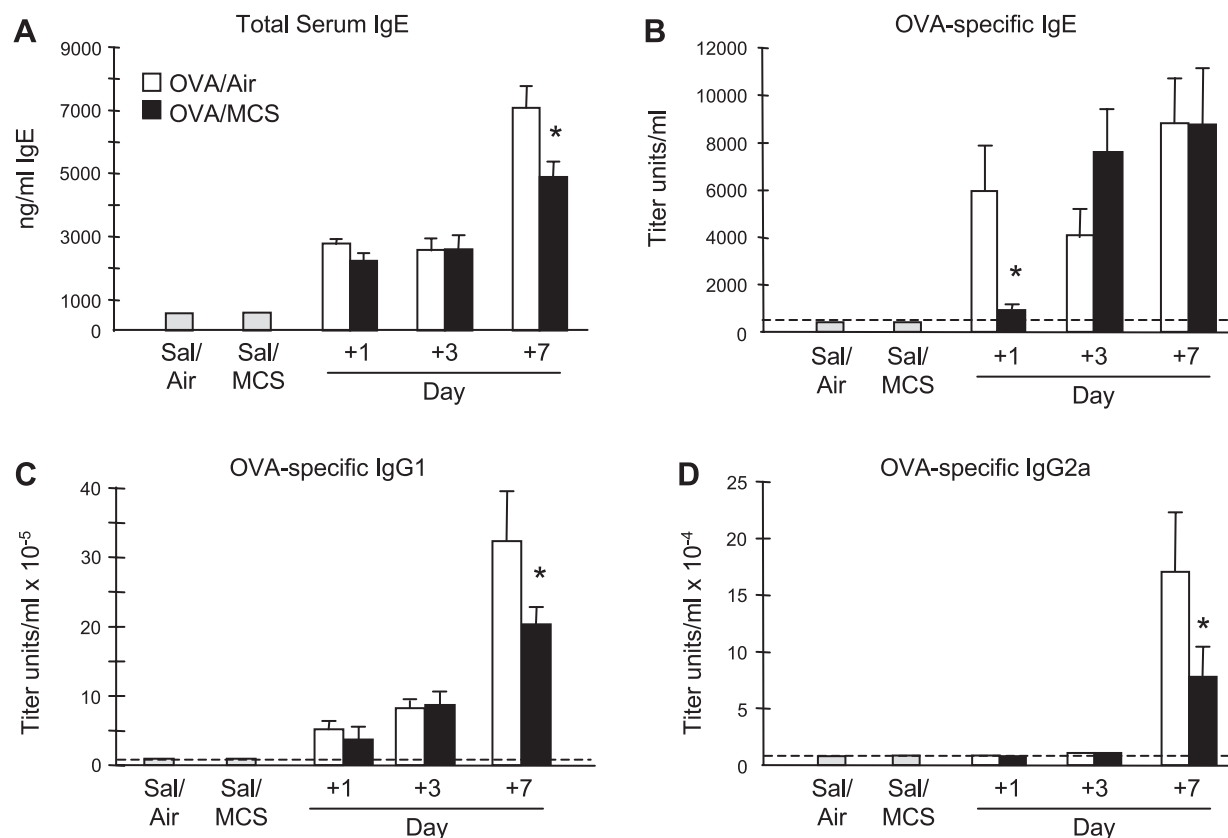


Fig. 6. Cigarette smoke exposure reduces the level of OVA-specific antibodies. Mice were sensitized to OVA or saline and exposed to air or smoke as described above. Total IgE (A) and OVA-specific IgE (B), IgG₁ (C), and IgG_{2a} (D) were measured in serum by isotype-specific ELISA. Results shown are the means $\pm$ SE for $n = 6$ mice per group and are representative of 2 independent experiments. *Significant decrease in MCS-exposed mice ($P < 0.05$, ANOVA). The dashed line indicates the lower limit of detection.

and IFN- γ was determined by ELISA, and proliferation was measured by [³H]thymidine incorporation assay. T cells harvested 3 and 7 days after OVA challenge produced both IL-4 and IFN- γ . Cytokine release was attenuated in T cells from mice that had been exposed to MCS (Fig. 7). Proliferation was also reduced in T cells from MCS-exposed mice harvested 1 day after OVA challenge but was unaffected in cells harvested 3 and 7 days after challenge (Fig. 8). Lymph nodes from mice exposed to saline and air, or saline and smoke, were small, and, as expected, the T cells did not respond to stimulation with OVA *in vitro* (data not shown).

Lower doses of MCS do not suppress immune sensitization. Although we report above that MCS exposure suppresses airway eosinophilia and OVA-specific T helper function, it has also been reported that cigarette smoke can act as an adjuvant to promote allergic sensitization (35, 36), a finding consistent with epidemiological studies. It has been suggested that second-hand smoke is chemically different and has adjuvant properties lacking in MCS (34). A simpler explanation is that higher exposures to MCS are immunosuppressive, whereas lower exposures promote immune responses. Groups of mice were sensitized to OVA and exposed daily to MCS as described above except that the number of cigarettes smoked and the dilution air flow rate were adjusted so that the final concentration of smoke in the exposure chamber was one-eighth of that previously used. OVA/MCS mice exhibited a slight reduction in BAL lymphocytes but no change in total

BAL cells, neutrophils, or eosinophils (Fig. 9). MCS exposure did not suppress the development of OVA-specific IgG₁ or IgE antibodies (Fig. 9, E and F).

DISCUSSION

Studies of the effects of MCS on allergic airway inflammation in mice have reported both attenuation (28, 34) and exacerbation (29, 36) of the allergic immune response. These studies cannot be directly compared due to differences in the strain of mice used, the route and manner of allergen sensitization, and the route and manner of MCS exposure. We have developed a MCS exposure system in which exposures are quantified and can be varied reproducibly and consistently (44, 45) and have used this system to investigate effects of different levels of MCS on allergic airways inflammation. We report here that MCS exposure attenuates the allergic inflammatory response by inhibiting T helper function in a dose-dependent manner.

We exposed mice to MCS (588 mg/m³ TSP for 2 h/day, estimated as the equivalent of 1–1½ packs per day; Ref. 27) following sensitization to OVA by intraperitoneal injection (Fig. 1). MCS alone provoked a significant inflammatory response dominated by neutrophils, whereas OVA alone provoked an eosinophilic inflammation typical of this model (Fig. 2). OVA/MCS mice exhibited decreased allergic inflammation, with reductions in BAL and tissue eosinophils. Inter-

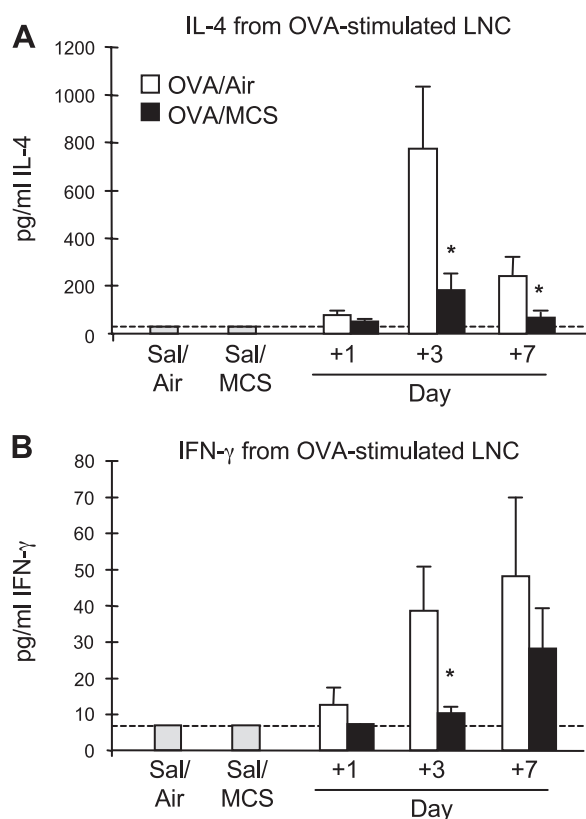


Fig. 7. Lymph node cells (LNC) from smoke-exposed mice express reduced cytokines on antigenic stimulation. Mice were sensitized to OVA or saline and exposed to air or smoke as described above. Following death, the peribronchial and mediastinal lymph nodes were harvested, and lymph node cells were cultured with 50 μ g/ml OVA. After 72 h of culture, IL-4 (A) and IFN- γ (B) in the culture medium were measured by ELISA. Prior in vivo exposure to cigarette smoke inhibits antigen-stimulated release of IL-4 and IFN- γ . *Significant decrease ($P < 0.05$, t -test).

estingly, the number of BAL neutrophils in OVA/MCS mice was reduced 75% on day +7 after challenge compared with both day +1 and to MCS alone. We hypothesize that the antigen-specific OVA response, being orchestrated by different cells and cytokines than the response to smoke, took priority over and dampened the inflammatory response to MCS.

MCS exposure also inhibited cytokine and immunoglobulin production after OVA challenge, with significant decreases in the Th2 cytokines IL-5 and IL-4 and OVA-specific antibodies. IL-5 is a key eosinophilic chemoattractant, and the reduction in IL-5 levels, especially at day +1, likely contribute to reduced migration of eosinophils into the lungs over the 7 days following OVA challenge. Similarly, the near absence of OVA-specific IgE antibodies on day +1 suggests that Th2 function necessary for B cell maturation and IgE class switching (15) was severely reduced in the OVA/MCS mice during the 2-wk MCS exposure period between OVA sensitization and challenge. As mucus production by airway epithelial cells is dependent on Th2 cytokines (6, 50), the dramatic reduction in goblet cell metaplasia in the OVA/MCS mice is another indication of reduced T helper function (Fig. 3, G and H). Finally, antigen-specific proliferation and production of both IL-4 and IFN- γ were reduced in T cells from OVA/MCS compared with OVA/Air mice. Taken together, these results suggest that MCS-exposed mice have reduced OVA-specific T helper func-

tion, especially early in the response. The reductions in IL-4 and mucus production might indicate that MCS exposure had altered the Th1/Th2 balance away from a Th2 allergic response. However, IgG_{2a}, which is typical of Th1 responses (43), was also reduced, and the level of IFN- γ in BALF and lung homogenates was too low to measure in these experiments, so a general inhibition of T helper function could not be ruled out. This is the first study to report that inhibition of the allergic airway response in mice exposed to a high dose of MCS is associated with decreased T helper function, contributing to reduced inflammation, reduced eosinophil recruitment, reduced goblet cell metaplasia, reduced production of antigen-specific antibodies, and delayed IgE class switching.

There are a number of potential mechanisms by which MCS exposure could suppress T cell function. Both nicotine and MCS suppress antigen-mediated signaling in T cells (20, 42), and MCS also decreases the number of dendritic cells (DCs) in lung tissue and impairs their activation (33). Therefore, the reduction in T cell effector function observed in this study could be due to direct effects of smoke on T cells themselves, to a reduction in T cell priming and stimulation resulting from suppression of DC function, or to both. Interestingly, a previous study reported that MCS inhibited the allergic airways response to OVA and reduced the number of T cells in the lung by 50% but increased production of T cell cytokines (34). In that study, mice were sensitized to OVA by exposing them to OVA aerosol in combination with intranasal administration of an adenoviral vector expressing granulocyte-macrophage colony-stimulating factor (GM-CSF). Since GM-CSF promotes the differentiation and maturation of DCs and enhances DC antigen-presentation (12, 51), this suggests that GM-CSF rescues MCS-impaired DCs and demonstrates that the route of allergen sensitization can significantly affect the outcome of these experiments.

It has also been reported that carbon monoxide (CO) impairs the allergic response to OVA (5). However, we feel that CO is not a significant contributor to the immune suppression in our

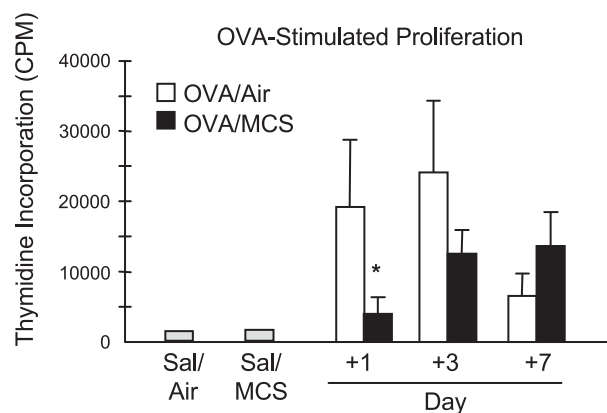


Fig. 8. Antigen-dependent lymph node cells proliferation in vitro is inhibited by prior exposure to cigarette smoke. Mice were sensitized to OVA or saline and exposed to air or smoke as described above. Following death, the peribronchial and mediastinal lymph nodes were harvested, and lymph node cells were cultured with 50 μ g/ml OVA. Proliferation was measured by [³H]thymidine incorporation as described. Prior in vivo exposure to cigarette smoke inhibits antigen-dependent proliferation 1 and 3 days after challenge. There were no differences in proliferation stimulated by concanavalin A, a nonspecific positive control stimulus. *Significant decrease ($P < 0.05$, ANOVA). CPM, counts per minute.

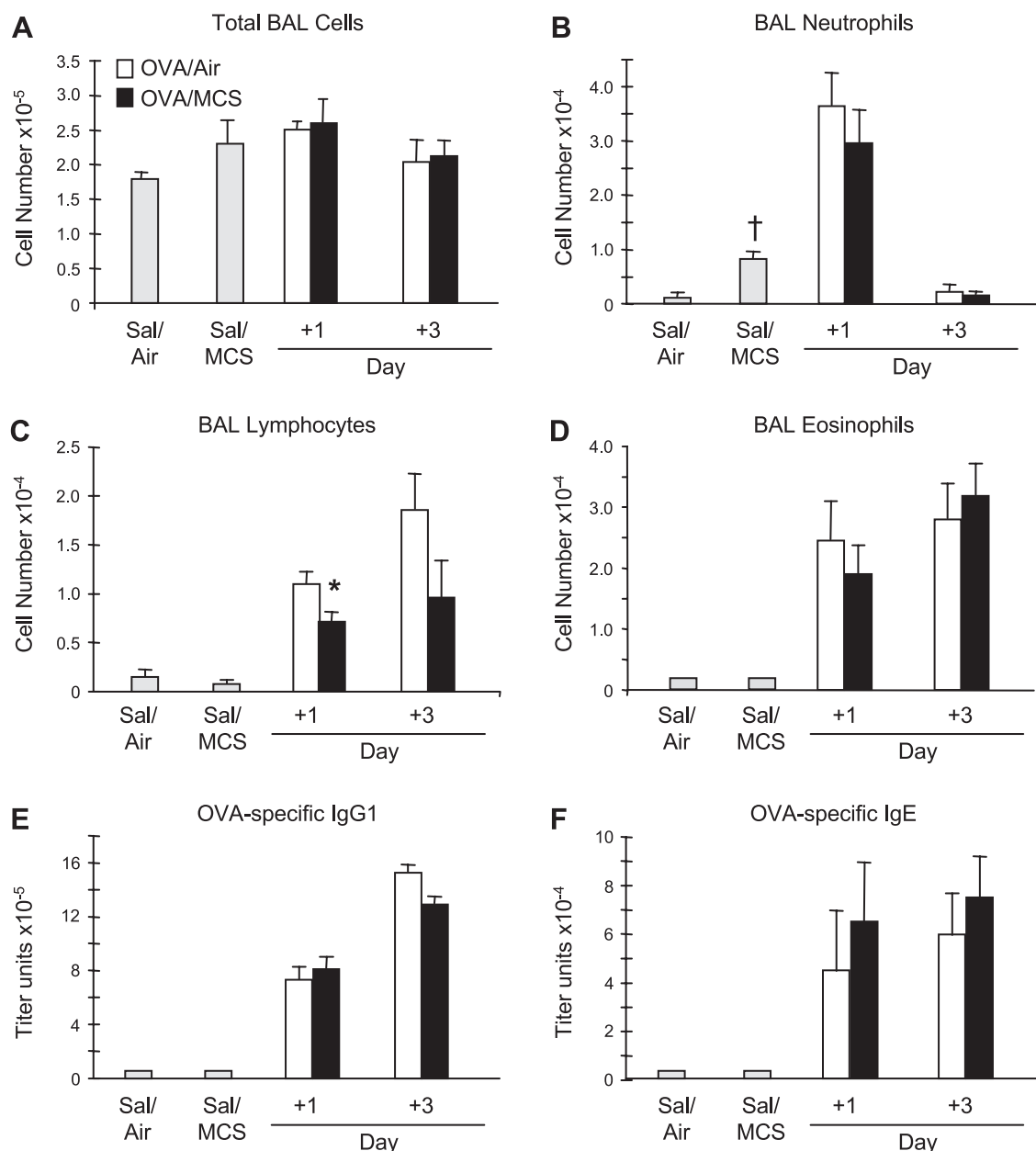


Fig. 9. Low-dose MCS exposure does not suppress the allergic response. Mice were sensitized to OVA and exposed to MCS as described in Fig. 1 except that the average MCS exposure was reduced by 83%. Mice were harvested on days 1 and 3 postchallenge. Differential counts of BAL cells are shown: total BAL cells (A), neutrophils (B), lymphocytes (C), and eosinophils (D). OVA-specific IgG₁ (E) and IgE (F) were measured in serum as described. Results shown are the means $\pm$ SE for $n = 5-6$ mice per group. †Significant increase in MCS-exposed mice over air-exposed; *significant decrease in OVA-sensitized/MCS-exposed mice compared with OVA-sensitized/air-exposed ($P < 0.05$, ANOVA).

experiments. We exposed mice to MCS containing CO [600 parts per million (ppm)] for 1 h. The half-life of carboxyhemoglobin (COHb) in mice is very short (48), such that COHb is undetectable in our mice after 30 min breathing room air (data not shown). In contrast, Chapman et al. (5) exposed mice to 250 ppm CO continuously for 48 h after antigen challenge. Furthermore, Chapman et al. (5) reported that CO significantly decreased BAL levels of PGE₂, whereas we have shown no difference in PGE₂ levels between OVA/Air and OVA/MCS-exposed mice (Fig. 5C).

Epidemiological studies have shown that ETS is a risk factor for the development of childhood asthma (14, 32), and animal

studies have reported that exposure of mice to very low doses of ETS enhances the allergic response to OVA (36) and to *Aspergillus fumigatus* (37). In contrast, we and others have also reported that MCS inhibits allergic airway inflammation in OVA-sensitized mice and has immunosuppressive properties against some human diseases (28, 34, 41). It has been suggested that this discrepancy is due to chemical differences between ETS and MCS (34). However, it should be noted that different laboratories use different MCS exposure methodologies, and significant parameters, including the duration of exposure, the amount of dilution air (if any), and the actual smoke concentration in the exposure chamber, are often unre-

ported (28, 29, 34, 35). We hypothesized that the different reported effects of MCS were due to quantitative, and not qualitative, differences in smoke exposure. To investigate this possibility, we reduced the number of cigarettes loaded on the carousel and increased the flow rate of dilution air into the exposure chamber, resulting in an 80% reduction in the MCS concentration without otherwise altering its physical or chemical properties. Mice were then sensitized to OVA and exposed to air or MCS before OVA challenge, exactly as described for the higher dose experiment. This level of exposure is still a potent proinflammatory stimulus. Acute exposure results in significant neutrophilic lung inflammation, with increased expression of proinflammatory cytokines including MIP-2, KC, TNF- α , and IL-6 (Ref. 54 and our unpublished data), and chronic exposure leads to prolonged accumulation of neutrophils (Fig. 9B). However, this level of MCS exposure did not suppress the response to OVA challenge, with no significant differences in BAL eosinophils or OVA-specific IgE and IgG₁ (Fig. 9). It is, of course, possible that there is an exposure level that would enhance the allergic response that we have not yet identified. It should also be noted that this study investigated the effects of MCS exposure on the effector phase of the allergic response. It has been reported that MCS or ETS exposure during the sensitization phase enhances allergic sensitization (35, 37), and we have found that exposure to OVA aerosol and low levels of MCS without prior immunization leads to sensitization rather than tolerance (data not shown). This suggests that cigarette smoke exposure may have different effects on the sensitization and effector phases of the response.

Our results demonstrate that MCS exposure inhibits the development of allergic airway inflammation by inhibiting antigen-specific T cell function in a dose-dependent manner. This may explain previous conflicting human and animal studies, in that exposure to low levels of ETS or MCS promotes allergic sensitization and asthma whereas high levels of MCS exposure associated with direct smoking promotes a systemic inflammatory response involved in cardiovascular and other diseases (24) but inhibits T cell-mediated responses involved in hypersensitivity pneumonitis, sarcoidosis, and clearance of respiratory viral infections (2). This also highlights the critical importance of accurate quantification of cigarette smoke exposures, both for human and animal studies, and the need to empirically determine smoke concentrations and thoroughly document smoking methodologies to ensure reproducibility of future results.

GRANTS

This research was supported, in part, by HL-088325, HL-075432, T32HL66988, T32ES07026, UL1RR024160, National Institute of Environmental Health Sciences Center Grant P30ES01247, and EPA R827354.

REFERENCES

1. al-Mehdi AB, Ischiropoulos H, Fisher AB. Endothelial cell oxidant generation during K⁺-induced membrane depolarization. *J Cell Physiol* 166: 274–280, 1996.
2. Arcavi L, Benowitz NL. Cigarette smoking and infection. *Arch Intern Med* 164: 2206–2216, 2004.
3. Baldwin CI, Todd A, Bourke S, Allen A, Calvert JE. Pigeon fanciers' lung: effects of smoking on serum and salivary antibody responses to pigeon antigens. *Clin Exp Immunol* 113: 166–172, 1998.
4. Bartal M. Health effects of tobacco use and exposure. *Monaldi Arch Chest Dis* 56: 545–554, 2001.
5. Chapman JT, Otterbein LE, Elias JA, Choi AM. Carbon monoxide attenuates aeroallergen-induced inflammation in mice. *Am J Physiol Lung Cell Mol Physiol* 281: L209–L216, 2001.
6. Cohn L, Homer RJ, MacLeod H, Mohrs M, Brombacher F, Bottomly K. Th2-induced airway mucus production is dependent on IL-4R α , but not on eosinophils. *J Immunol* 162: 6178–6183, 1999.
7. Di Stefano A, Caramori G, Ricciardolo FL, Capelli A, Adcock IM, Donner CF. Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. *Clin Exp Allergy* 34: 1156–1167, 2004.
8. Eisner MD. Environmental tobacco smoke and adult asthma. *Clin Chest Med* 23: 749–761, 2002.
9. Eisner MD, Iribarren C. The influence of cigarette smoking on adult asthma outcomes. *Nicotine Tob Res* 9: 53–56, 2007.
10. Eisner MD, Klein J, Hammond SK, Koren G, Lactao G, Iribarren C. Directly measured second hand smoke exposure and asthma health outcomes. *Thorax* 60: 814–821, 2005.
11. Eliasson B. Cigarette smoking and diabetes. *Prog Cardiovasc Dis* 45: 405–413, 2003.
12. Fleetwood AJ, Cook AD, Hamilton JA. Functions of granulocyte-macrophage colony-stimulating factor. *Crit Rev Immunol* 25: 405–428, 2005.
13. Gergen PJ, Fowler JA, Maurer KR, Davis WW, Overpeck MD. The burden of environmental tobacco smoke exposure on the respiratory health of children 2 months through 5 years of age in the United States: Third National Health and Nutrition Examination Survey, 1988 to 1994. *Pediatrics* 101: E8, 1998.
14. Goodwin RD. Environmental tobacco smoke and the epidemic of asthma in children: the role of cigarette use. *Ann Allergy Asthma Immunol* 98: 447–454, 2007.
15. Gould HJ, Sutton BJ, Beavil AJ, Beavil RL, McCloskey N, Coker HA, Fear D, Smurthwaite L. The biology of IGE and the basis of allergic disease. *Annu Rev Immunol* 21: 579–628, 2003.
16. Harkema JR, Mariassy AT, St. George J, Hyde DM, Plopper CG. Epithelial cells of the conducting airways: a species comparison. In: *The Airway Epithelium: Physiology, Pathophysiology and Pharmacology*, edited by Farmer SG and Hay DWP. New York: Dekker, 1991, p. 3–39.
17. Hjern A, Hedberg A, Haglund B, Rosen M. Does tobacco smoke prevent atopic disorders? A study of two generations of Swedish residents. *Clin Exp Allergy* 31: 908–914, 2001.
18. Jarvis D, Chinn S, Luczynska C, Burney P. The association of smoking with sensitization to common environmental allergens: results from the European Community Respiratory Health Survey. *J Allergy Clin Immunol* 104: 934–940, 1999.
19. Jarvis D, Luczynska C, Chinn S, Burney P. The association of age, gender and smoking with total IgE and specific IgE. *Clin Exp Allergy* 25: 1083–1091, 1995.
20. Kalra R, Singh SP, Savage SM, Finch GL, Sopori ML. Effects of cigarette smoke on immune response: chronic exposure to cigarette smoke impairs antigen-mediated signaling in T cells and depletes IP3-sensitive Ca²⁺ stores. *J Pharmacol Exp Ther* 293: 166–171, 2000.
21. Kamholz SL. Pulmonary and cardiovascular consequences of smoking. *Clin Occup Environ Med* 5: 157–171, x, 2006.
22. Koumas L, King AE, Critchley HO, Kelly RW, Phipps RP. Fibroblast heterogeneity: existence of functionally distinct Thy 1⁺ and Thy 1[−] human female reproductive tract fibroblasts. *Am J Pathol* 159: 925–935, 2001.
23. Long JA, Fogel-Petrovic M, Knight DA, Thompson PJ, Upham JW. Higher prostaglandin E₂ production by dendritic cells from subjects with asthma compared with normal subjects. *Am J Respir Crit Care Med* 170: 485–491, 2004.
24. MacCallum PK. Markers of hemostasis and systemic inflammation in heart disease and atherosclerosis in smokers. *Proc Am Thorac Soc* 2: 34–43, 2005.
25. Mallampalli A, Guntupalli KK. Smoking and systemic disease. *Clin Occup Environ Med* 5: 173–192, x, 2006.
26. Martey CA, Pollock SJ, Turner CK, O'Reilly KM, Baglote CJ, Phipps RP, Sime PJ. Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E₂ synthase in human lung fibroblasts: implications for lung inflammation and cancer. *Am J Physiol Lung Cell Mol Physiol* 287: L981–L991, 2004.
27. Mauderly JL, Gigliotti AP, Barr EB, Bechtold WE, Belinsky SA, Hahn FF, Hobbs CA, March TH, Seilkop SK, Finch GL. Chronic inhalation exposure to mainstream cigarette smoke increases lung and nasal tumor incidence in rats. *Toxicol Sci* 81: 280–292, 2004.

28. Melgert BN, Postma DS, Geerlings M, Luinge MA, Klok PA, van der Strate BW, Kerstjens HA, Timens W, Hylkema MN. Short-term smoke exposure attenuates ovalbumin-induced airway inflammation in allergic mice. *Am J Respir Cell Mol Biol* 30: 880–885, 2004.
29. Moerloose KB, Pauwels RA, Joos GF. Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. *Am J Respir Crit Care Med* 172: 168–172, 2005.
30. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. *Respir Res* 7: 49, 2006.
31. Murin S, Bilello KS, Matthay R. Other smoking-affected pulmonary diseases. *Clin Chest Med* 21: 121–137, ix, 2000.
32. Raherison C, Penard-Morand C, Moreau D, Caillaud D, Charpin D, Kopfersmitt C, Lavaud F, Taytard A, Annesi-maesano I. In utero and childhood exposure to parental tobacco smoke, and allergies in school-children. *Respir Med* 101: 107–117, 2007.
33. Robbins CS, Dawe DE, Goncharova SI, Pouladi MA, Drannik AG, Swirski FK, Cox G, Stampfli MR. Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. *Am J Respir Cell Mol Biol* 30: 202–211, 2004.
34. Robbins CS, Pouladi MA, Fattouh R, Dawe DE, Vujicic N, Richards CD, Jordana M, Inman MD, Stampfli MR. Mainstream cigarette smoke exposure attenuates airway immune inflammatory responses to surrogate and common environmental allergens in mice, despite evidence of increased systemic sensitization. *J Immunol* 175: 2834–2842, 2005.
35. Rumold R, Jyrälä M, Diaz-Sanchez D. Secondhand smoke induces allergic sensitization in mice. *J Immunol* 167: 4765–4770, 2001.
36. Seymour BW, Pinkerton KE, Frieberthausen KE, Coffman RL, Gershwin LJ. Second-hand smoke is an adjuvant for T helper-2 responses in a murine model of allergy. *J Immunol* 159: 6169–6175, 1997.
37. Seymour BW, Schelegle ES, Pinkerton KE, Frieberthausen KE, Peake JL, Kurup VP, Coffman RL, Gershwin LJ. Second-hand smoke increases bronchial hyperreactivity and eosinophilia in a murine model of allergic aspergillosis. *Clin Dev Immunol* 10: 35–42, 2003.
38. Siroux V, Pin I, Oryszczyn MP, Le Moual N, Kauffmann F. Relationships of active smoking to asthma and asthma severity in the EGEA study. Epidemiological study on the Genetics and Environment of Asthma. *Eur Respir J* 15: 470–477, 2000.
39. Smith CJ, Perfetti TA, King JA. Perspectives on pulmonary inflammation and lung cancer risk in cigarette smokers. *Inhal Toxicol* 18: 667–677, 2006.
40. Snijdwint FG, Kalinski P, Wierenga EA, Bos JD, Kapsenberg ML. Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J Immunol* 150: 5321–5329, 1993.
41. Sopori M. Effects of cigarette smoke on the immune system. *Nat Rev Immunol* 2: 372–377, 2002.
42. Sopori ML, Cherian S, Chilukuri R, Shopp GM. Cigarette smoke causes inhibition of the immune response to intratracheally administered antigens. *Toxicol Appl Pharmacol* 97: 489–499, 1989.
43. Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, Vitetta ES. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 334: 255–258, 1988.
44. Thatcher TH, Maggirwar SB, Baglolo CJ, Lakatos HF, Gasiewicz TA, Phipps RP, Sime PJ. Aryl hydrocarbon receptor-deficient mice develop heightened inflammatory responses to cigarette smoke and endotoxin associated with rapid loss of the nuclear factor- κ B component RelB. *Am J Pathol* 170: 855–864, 2007.
45. Thatcher TH, McHugh NA, Egan RW, Chapman RW, Hey JA, Turner CK, Redonnet MR, Seweryniak KE, Sime PJ, Phipps RP. Role of CXCR2 in cigarette smoke-induced lung inflammation. *Am J Physiol Lung Cell Mol Physiol* 289: L322–L328, 2005.
46. Thatcher TH, O'Brien DP, Altuwaijri S, Barth RK. Increasing the frequency of T-cell precursors specific for a cryptic epitope of hen-egg lysozyme converts it to an immunodominant epitope. *Immunology* 99: 235–242, 2000.
47. Varga SM, Beckman NA, Chu M, Braciale TJ. Sensitive detection and quantitation of mouse eosinophils in tissues using an enzymatic eosinophil peroxidase assay: its use to rapidly measure pulmonary eosinophilia during experimental respiratory syncytial virus infection of mice. *J Immunol Methods* 262: 111–120, 2002.
48. Watson ES, Jones AB, Ashfaq MK, Barrett JT. Spectrophotometric evaluation of carboxyhemoglobin in blood of mice after exposure to marijuana or tobacco smoke in a modified Walton horizontal smoke exposure machine. *J Anal Toxicol* 11: 19–23, 1987.
49. Weiss ST, Utell MJ, Samet JM. Environmental tobacco smoke exposure and asthma in adults. *Environ Health Perspect* 107, Suppl 6: 891–895, 1999.
50. Whittaker L, Niu N, Temann UA, Stoddard A, Flavell RA, Ray A, Homer RJ, Cohn L. Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9. *Am J Respir Cell Mol Biol* 27: 593–602, 2002.
51. Williams MA, Kelsey SM, Newland AC. GM-CSF and stimulation of monocyte/macrophage function in vivo relevance and in vitro observations. *Eur J Cancer* 35, Suppl 3: S18–S22, 1999.
52. Yanbaeva DG, Dentener MA, Creutzberg EC, Wesseling G, Wouters EF. Systemic effects of smoking. *Chest* 131: 1557–1566, 2007.
53. Yang SR, Chida AS, Bauter MR, Shafiq N, Seweryniak K, Maggirwar SB, Kilty I, Rahman I. Cigarette smoke induces proinflammatory cytokine release by activation of NF- κ B and posttranslational modifications of histone deacetylase in macrophages. *Am J Physiol Lung Cell Mol Physiol* 291: L46–L57, 2006.
54. Yao H, Edirisinghe I, Rajendrasozhan S, Yang SR, Caito S, Adenuga D, Rahman I. Cigarette smoke-mediated inflammatory and oxidative responses are strain-dependent in mice. *Am J Physiol Lung Cell Mol Physiol* 294: L1174–L1186, 2008.