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Allergic asthma, an inflammatory disease characterized by the infiltration and activation of various leukocytes, the production of Th2 cytokines and leukotrienes, and atopy, also affects the function of other cell types, causing goblet cell hyperplasia/hyperactivity, increased mucus production/secretion, and airway hyperreactivity. Eosinophilic inflammation is a characteristic feature of human asthma, and recent evidence suggests that eosinophils also play a critical role in T cell trafficking in animal models of asthma. Nicotine is an anti-inflammatory, but the association between smoking and asthma is highly contentious and some report that smoking cessation increases the risk of asthma in ex-smokers. To ascertain the effects of nicotine on allergy/asthma, Brown Norway rats were treated with nicotine and sensitized and challenged with allergens. The results unequivocally show that, even after multiple allergen sensitizations, nicotine dramatically suppresses inflammatory/allergic parameters in the lung including the following: eosinophilic/lymphocytic emigration; mRNA and/or protein expression of the Th2 cytokines/chemokines IL-4, IL-5, IL-13, IL-25, and eotaxin; leukotriene C4; and total as well as allergen-specific IgE. Although nicotine did not significantly affect hexosaminidase release, IgG, or methacholine-induced airway resistance, it significantly decreased mucus content in bronchoalveolar lavage; interestingly, however, despite the strong suppression of IL-4/IL-13, nicotine significantly increased the intraepithelial-stored mucosubstances and Muc5ac mRNA expression. These results suggest that nicotine modulates allergy/asthma primarily by suppressing eosinophil trafficking and suppressing Th2 cytokine/chemokine responses without reducing goblet cell metaplasia or mucous production and may explain the lower risk of allergic diseases in smokers. To our knowledge this is the first direct evidence that nicotine modulates allergic responses. The Journal of Immunology, 2008, 180: 7655–7663.

The prevalence and morbidity of asthma and other allergic diseases, particularly in developed nations, have increased dramatically during the past several decades (1). Smoking is associated with a number of adverse health effects in humans, including significantly higher risks for developing lung cancer, chronic obstructive pulmonary disease, and respiratory tract infections (2). Several longitudinal and cross-sectional epidemiological studies suggest an inverse correlation between tobacco smoking and the development of some allergic and inflammatory diseases. In one longitudinal study, smokers showed significantly lower allergic skin reactivity than nonsmokers or ex-smokers (3, 4). Although smokers have higher serum IgE levels, they have lower aeroallergen-specific IgE and hay fever (5–7). Similarly, the incidence of hypersensitivity allergic diseases such as farmer’s lung and pigeon breeder’s disease is lower among smokers (8–11). Although the effects of smoking on the development of asthma are controversial (12, 13), several studies suggest that the risk of rhinitis/asthma is lower among current smokers than nonsmokers or ex-smokers. Baldacci et al. (14) observed that “never” smokers had a higher incidence of asthma and rhinitis than current smokers, and the incidence of asthma was higher among “never” and nonsmoking women than women who continued to smoke (15). Furthermore, the symptoms of asthma and bronchitis were more prevalent among nonsmoking iron miners than iron miners who smoked (16). In a large Danish study with >10,000 subjects, current smokers had a lower chance of developing asthma than ex-smokers (17); among Japanese agriculture workers smoking cessation was significantly associated with the activation of latent farmer’s lung (18). Thus, many studies suggest that smoking lowers the risk of allergy/hypersensitivity diseases; however, the mechanism by which cigarette smoke attenuates allergy/asthma is unclear.

Allergic asthma is an inflammatory lung disease involving the participation of several cell types, including leukocytes and airway epithelial and smooth muscle cells. The disease is characterized by pronounced infiltration of eosinophils and T cells into the submucosal tissues of airways, mucous cell hyperplasia/hyperactivity, increased mucus production, airway hyperresponsiveness (AHR),

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2 Address correspondence and reprint requests to Dr. Mohan L. Sopori, Lovelace Respiratory Research Institute, 2425 Ridgcrest Drive Southeast, Albuquerque, NM 87108. E-mail address: msopori@lrr.org
3 Abbreviations used in this paper: AHR, airway hyperresponsiveness; AB-PAS, Alcian Blue and periodic acid-Schiff solution; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; BN, Brown Norway; Ct, threshold cycle; CON, control; GABA, γ-aminobutyric acid; HDM, house dust mite; IHC, immunohistochemistry; i.t., intratracheal(ly); LTC4, leukotriene C4; NRW, NT treated and RW sensitized/challenged; NT, nicotine; qPCR, real-time quantitative PCR; RW, ragweed.

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airway remodeling, and elevated production of total and allergen-specific IgE (19, 20). Although the importance of eosinophilic accumulation in human asthma has been well established, recent evidence suggests that eosinophils are also critical in airway pathology and Th2 responses in the mouse OVA model of asthma (21). There is a strong correlation between allergic asthma and the presence of Th2-regulated cytokines/chemokines such as IL-4, IL-5, IL-13, IL-25, and eotaxin (22, 23). Moreover, IL-5 and eotaxin may have an important role in eosinophilia and human allergic asthma (24, 25). Indeed, double transgenic mice expressing IL-5 systemically and eotaxin in the lung develop several pulmonary pathologies representative of severe asthma (26). Interestingly, Th2-mediated diseases such as ulcerative colitis, endometriosis, and allergic rhinitis are relatively uncommon in smokers (27–29). Nicotine (NT), the major constituent of cigarette smoke, suppresses adaptive and inflammatory immune responses (30–32). In this communication we present evidence that NT attenuates aerosol allergen sensitization by primarily blocking the Th2 responses in the lung.

Materials and Methods

Animals

Six- to 8-wk-old, pathogen-free, female Brown Norway (BN) rats were purchased from Charles River Laboratories. Animals were kept in shoe box cages with hardwood chip bedding and in class 100 air quality rooms. Food and water were provided ad libidum throughout the experimental period. Animals were periodically monitored for common rat infections. All studies were approved by the Institutional Animal Care and Use Committee of Lovelace Respiratory Research Institute (Albuquerque, NM).

NT treatment

Rats were anesthetized with isoflurane-oxygen, and a 28-day constant release miniosmotic pumps (Alzet) were implanted s.c. in the backs of the necks of the animals (33). The pumps delivered saline (control (CON)) or −1 mg of NT per kg of body weight per day to achieve a plasma NT concentration of ≈28 ng/ml that is approximately equivalent to the plasma NT level of a one-pack-per-day human smoker (34).

Allergen sensitization and challenge

Seven days after NT/saline treatment, rats were sensitized i.p. (day 0) with 200 μg of endotoxin-free (endotoxin < 2.3 ng/mg) ragweed (RW) Ag or house dust mite (HDM; Greer Laboratories) mixed with 30 μl of alum (45 mg/ml aluminum hydroxide and 40 mg/ml magnesium hydroxide; Pierce) and 70 μl of Coca’s buffer (85 mM NaCl and 64 mM NaHCO3 (pH 8.1)). The sensitization procedure was repeated after 4 days (day 4). One week after the second sensitization (day 11), these rats were anesthetized with isoflurane-oxygen and challenged by an intratracheal (i.t.) instillation of 16 μg of RW or HDM in 100 μl of PBS. For the multiple challenges protocol, rats were sensitized twice (day 0 and day 4) as described above and challenged three times through i.t. delivery (days 11–13) of 16 μg of RW. The main reason for using the multiple allergen challenge protocol was that the results from early experiments had indicated a virtual block of allergen-induced changes by nicotine in the single allergen-challenged animals. It has been shown that multiple exposure to an allergen incrementally increased allergic responses in BN rats (35). Therefore, unless indicated otherwise, we have used multiple allergen challenges in these experiments.

Bronchoalveolar lavage (BAL) and cell collection

The trachea of each rat was surgically exposed, cannulated, and tied off with a silk thread suture. The left lobe was tied off to prepare for histology, and the right lobe was lavaged twice with 2.5 ml of saline. Lavages were pooled, and BAL cells were collected by centrifugation, resuspended in PBS, and counted. Viability was assessed by trypan blue exclusion. Approximately 50,000 cells from each sample were centrifuged onto duplicate cytospin slides and stained with Diff-Quik (Baxter Healthcare) to score eosinophils, macrophages, neutrophils, and lymphocytes. At least 200 cells per slide were counted to obtain the differential leukocyte count.

Lung histopathology

The left lung lobe was inflated and fixed with −5 ml of 4% (v/v) paraformaldehyde in deionized water (Sigma-Aldrich), immersed in 4% paraformaldehyde for 24 h, and then transferred into Tris-buffered saline (pH 7.4). Lungs were trimmed in the dorsoventral transverse direction from cranial region to the caudal region and the tissue was embedded in paraffin. Five-micrometer-thick sections were cut from the tissues, stained with H&E, and read by the pathologist in a blinded manner. The degree of pulmonary inflammation was graded on a subjective scale from 0 to 5, based upon the severity and distribution of inflammation.

Cytokine and leukotriene C4 (LTC4) levels in BAL

The concentrations of IL-4 and IL-13 in BAL fluids (BALF) were determined by rat ELISA Cytoscreen kits (BioSource International). The LTC4 content was determined by an ELISA kit (Cayman Chemicals) according to the manufacturer’s directions and as described elsewhere (36).

Determination of total and allergen-specific IgE and IgG

RW-specific Ig production was measured by ELISA as described (35, 37). Briefly, for RW-specific IgE and IgG assays, 96-well flat-bottom Immunonol 4 microtiter ELISA plates (Dynex Laboratories) were coated with 20 μg/ml RW Ag in 0.1 M carbonate buffer (pH 9.5) and incubated overnight at room temperature. Plates were then saturated (blocked) with 2% gelatin in PBS with 0.05% Tween 20 and incubated for 2 h at 37°C. To test the IgE, 1/10 and 1/100 dilutions of test serum were added to the wells and incubated overnight at room temperature. To measure RW-specific IgG and IgG subclasses (IgG1 and IgG2a), 1/100 dilution (lavage) and 1/1000 dilution (serum) were used. IgE was detected by a 1/1000 dilution of a
Table I. Average lung inflammation scores in RW-sensitized BN rats with and without NT pre-treatment

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Eosinophilic and Histiocytic/Granulomatous Inflammation Average (SD)</th>
<th>Eosinophilic Peribronchovascular Inflammation Average (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>2 (0)</td>
<td>1.7 (0.6)</td>
</tr>
<tr>
<td>RW</td>
<td>4 (0)</td>
<td>2.7 (0.6)</td>
</tr>
<tr>
<td>NRW</td>
<td>1.3 (1.1)</td>
<td>1.7 (0.6)</td>
</tr>
</tbody>
</table>

biodin-conjugated mouse anti-rat IgE (BD Pharmingen) for 2 h at the ambient temperature followed by a 1/1000 dilution of HRP-conjugated avidin (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. IgG, IgG1, and IgG2a were detected by a 1/1000 dilution of biotin-conjugated goat anti-rat IgG (BD Pharmingen) and mouse anti-rat IgG1 and IgG2a (BD Biosciences) for 2 h at the ambient temperature. In the final step, 100 µl/well HRP substrate (BD Biosciences/BD Pharmingen) was added and reactions developed at room temperature for at least 10 min to detect IgE, IgG, and IgG subclasses. The reaction was stopped by the addition of 50 µl of 2 N NaOH, and the OD of the samples was determined at 405 nm by a Spectromax ELISA plate reader (Molecular Devices).

Real-time quantitative PCR (qPCR)

Total RNA was isolated from lung tissues using TRI Reagent (Molecular Research Center) as described elsewhere (29). Briefly, lung tissues were homogenized in 1 ml of TRI Reagent, 100 µl of 1-bromo-3-chloropropane (BCP; Molecular Research Center) was added, and then the tissues were centrifuged at 13,000 × g for 10 min at 4°C. The aqueous layer was collected and mixed with 600 µl of isopropanol. After 15 min at room temperature, samples were centrifuged (13,000 × g for 10 min) and the pellet was resuspended in 75% ethanol, centrifuged as described above, and then air dried. The samples were then resuspended in diethylpyrocarbonate-treated water (55°C for 10 min to dissolve RNA) and quantified spectrophotometrically. qPCR analysis was performed on the ABI Prism 7900HT real-time PCR System using the One-Step RT-PCR Master Mix (Applied Biosystems). The threshold cycle (Ct), which is defined as the cycle at which PCR amplification reaches a significant value (i.e., usually 15 times greater than the SD of the baseline), is given as the mean value. The relative expression of each mRNA was calculated by the ∆∆Ct method (where ∆∆Ct is the value obtained by subtracting the Ct value of control from the Ct value of the target mRNA). Specifically, the amount of target mRNA relative to control mRNA is expressed as 2^(-∆∆Ct). Data are expressed as the ratio of the target mRNA to control mRNA. Because all results were derived from the linear amplification curve, the use of ∆∆Ct method ensures that only mRNA amplification within the linear range is measured. Specific primer/probe sets for IL-13, IL-4, IL-5, 18S, and GAPDH were purchased from Applied Biosystems.

For Muc5ac qPCR, amplification was performed under the following conditions: PCR was performed at 95°C for 15 min and 40 cycles at 95°C for 15 s, 60°C for 20 s, and 74°C for 20 s. PCR detection Ct values were calculated by ABI Prism 7900HT Sequence Detection System Software (SDS2.1; Applied Biosystems), and differences between samples were calculated using the 2^(-∆∆Ct) method. The following primers were used: 5'-TACAATGGGCAACGGTACCATCCT-3' (forward) and 5'-AACGGTACCATCCT-3' (reverse). Eotaxin mRNA expression in the lung tissue was determined on the ABI Prism 7900HT real-time PCR System using the One-Step RT-PCR Master Mix (Applied Biosystems). The threshold cycle (Ct), which is defined as the cycle at which PCR amplification reaches a significant value (i.e., usually 15 times greater than the SD of the baseline), is given as the mean value. The relative expression of each mRNA was calculated by the ∆∆Ct method (where ∆∆Ct is the value obtained by subtracting the Ct value of control from the Ct value of the target mRNA). Specifically, the amount of target mRNA relative to control mRNA is expressed as 2^(-∆∆Ct). Data are expressed as the ratio of the target mRNA to control mRNA. Because all results were derived from the linear amplification curve, the use of ∆∆Ct method ensures that only mRNA amplification within the linear range is measured. Specific primer/probe sets for IL-13, IL-4, IL-5, 18S, and GAPDH were purchased from Applied Biosystems.

Immunohistochemistry

The endogenous peroxidase was blocked by incubating the slides containing tissue sections in 2% hydrogen peroxide/methanol for 1 min. The slides were washed with deionized water followed by washes with Dulbecco’s PBS (pH 7.4) containing 0.05% Brij. Proteins were unmasked by incubating tissue sections with a trypsin solution (Zymed Laboratories) at 37°C for 10 min. After blocking nonspecific binding by 1/10 horse serum containing 2% BSA and 0.1% Triton X-100, the slides were incubated overnight at 4°C in 1/100 diluted polyclonal goat anti-mouse IL-25 Ab (Santa Cruz Biotechnology). For eotaxin, tissue sections were incubated with a 1/50
dilution of goat anti-mouse eotaxin Ab (R&D Systems) and slides were developed with biotinylated rabbit anti-goat Ab, the Vectastain ABC reagent, and the peroxidase substrate diaminobenzidine (Vector Laboratories) according to the manufacturer’s instructions.

Morphometry for mucous cell numbers and intraepithelial mucosubstance

The intrapulmonary airways of the left lung lobe from each animal were microdissected under a high-resolution microscope. Beginning at the lobar bronchus, the airways were split down the long axis of the axial pathway through the 11th airway generation. Three-millimeter-thick lung slices at the level of the 5th (proximal) and 11th (distal) generation airways were embedded in paraffin and cut into 5-μm-thick sections. The tissue sections were stained with Alcian blue and periodic acid-Schiff solution (AB-PAS) as previously described (38). The number of AB-PAS-stained mucus cells per millimeter of basal lamina and the mucus volume per cubic meter of basement membrane in tissue sections were quantified blinded using an Olympus BH-2 light microscope equipped with the National Institutes of Health image analysis system as described previously (39).

Muc5ac ELISA in BALF

Muc5ac protein secretion in the BALF was determined by ELISA using the Muc5ac-specific mAb 45M1 (New Markers) as described previously (40). Briefly, 50-μl aliquots of BALF were serially diluted in 50 mM bicarbonate buffer (pH 9.5) and dried in 96-well immunoplates (MaxiSorp surface; Nalge Nunc International) at 40°C. Samples were blocked with 2% BSA and sequentially incubated at room temperature for 1 h with the 45M1 mAb (50 μl/well, 1:500), peroxidase-labeled goat anti-mouse Ab (100 μl/well at 1/5,000; Kirkegaard & Perry. The relative amount of Muc5ac was determined by absorbance at 450 nm with the standard curve.

Measurement of airway resistance

Twenty-four hours after the last i.t. challenge with either saline or RW, airway resistance was measured by plethysmography using the flexiVent system (SCIREQ Scientific Respiratory Equipment) as previously described (41). Briefly, rats were anesthetized by an i.p. injection of Avertin (250 mg/kg). The animals were intubated and placed on the flexiVent system, and airway resistance was measured at increasing doses of aerosolized methacholine (0, 1, 3, 6, 12, 25, and 50 mg/ml). Values for lung resistance were obtained at 5-s intervals for 3 min after each methacholine challenge. The peak responses at each methacholine concentration were used for data analysis.

Statistical analysis

The data were analyzed by Graph Pad Prism software 3.0 (GraphPad Software) using the Student’s t test or by two-way ANOVA. Results are presented as the mean ± SE of the combined experiments. The differences with p ≤ 0.05 were considered significant.
Results
Nicotine inhibits ragweed-induced influx of leukocytes into the lung

Airway allergic sensitization is associated with the inflammation and recruitment of lymphocytes, mast cells, and eosinophils into the lung. To determine the effects of NT treatment on lung inflammation, H&E-stained lung sections from saline- and NT-treated rats were examined microscopically after sensitization/challenge with RW. As in humans, normal BN rats exhibit a moderate baseline leukocytic infiltration in the lung (35). Compared with unsensitized controls (Fig. 1, A and B), RW induced a marked increase in the eosinophilic and histiocytic granulomatous interstitial inflammation as well as eosinophilic peribronchovascular inflammation in the lungs of sensitized BN rats (Fig. 1, C and D). An increase in lymphocytes was also present in the inflammatory foci of these rats (Fig. 1D). In contrast, compared with unsensitized rats, animals pretreated with nicotine before RW sensitization did not show significant increase in lung inflammation (Fig. 1, E and F). Results from a semiquantitative histological evaluation of animals from various treatment groups are summarized in Table I. NT by itself has no significant effect on any basal (unsensitized) lung parameters tested in these studies (not shown). Differential cell count indicated that NT inhibited the RW-induced accumulation of leukocytes (Fig. 2A) and eosinophils (Fig. 2B) in BALF. Similar inhibitory effects of NT were also seen on the leukocytic infiltration as well as the accumulation lymphocytes in the lungs of HDM Ag-treated animals (data not shown). Thus, NT treatment blunts the lung inflammatory response associated with allergen sensitization.

Nicotine suppresses total and allergen-specific IgE, but not IgG

IgE provides the biological basis for allergy and plays an important role in bronchial hyperreactivity. In contrast, allergen-specific IgG is usually protective (42). To ascertain the effects of NT treatment on allergen-induced IgE and IgG, RW-sensitized/challenged and NT-treated, RW-sensitized/challenged (NRW) rats were sacrificed 24 h after i.t. challenge. BAL and serum levels of IgE, IgG, and IgG subclasses (IgG1 and IgG2a) of the RW and NRW groups were compared with CON animals. It is clear that RW increased the total and RW-specific IgE in the BAL (Fig. 3, A and B) and serum (Fig. 3, C and D). NT significantly decreased RW-specific IgE in the BAL and serum and decreased total IgE in the BAL, but not in the serum. However, NT significantly inhibited the increase in total IgE in the BAL and serum in HDM-sensitized/challenged animals (Fig. 3, E and F). On the contrary, NT had no significant effect on the RW-specific IgG levels in the BAL (Fig. 4A) or serum (Fig. 4B); in fact, the RW-specific IgG was somewhat increased in the BAL by NT treatment. Similarly, neither the BAL (not shown) nor the serum levels of RW-specific IgG1 and IgG2a were significantly affected by NT treatment (Figs. 4, C and D). Moreover, the
total serum level of IgG was essentially unaffected by NT treatment (not shown). These results suggest that NT suppresses allergen-induced increases in IgE but not in IgG or IgG subclasses in the BALF and serum.

Nicotine blocks RW-induced LTC\textsubscript{4} secretion

The cysteinyl leukotriene LTC\textsubscript{4}, a powerful mediator of bronchoconstriction, is produced by a number of inflammatory cells, particularly mast cells, in allergic asthma (43). To determine whether NT affects the production of LTC\textsubscript{4} in the lung, BAL levels from CON rats were compared with those sensitized/challenged with RW and the animals treated with NT and then sensitized/challenged with RW, the NRW rats. Fig. 5 shows that NT treatment essentially blocked the production of LTC\textsubscript{4} in the BAL after RW challenge. Thus, NT suppresses the lung production of a potent bronchoconstricting leukotriene in response to allergens.

Nicotine strongly down-regulates allergen-induced Th2 cytokines

A strong correlation exists between the development of allergic asthma and elevated levels of Th2 cytokines and chemokines, primarily IL-4, IL-5, IL-13, IL-25, and eotaxin (22, 23, 25, 44). To ascertain the effects of NT on the production of these cytokines, their lung expression was determined by qPCR or immunohistochemistry (IHC) and the BAL content of some of these cytokines was determined by ELISA. In these experiments, after RW sensitization an i.t. allergen challenge was given either once (day 11) or on three consecutive days (days 11–13). Although a single challenge with RW in RW-sensitized animals caused a robust increase in the expression of Th2 cytokines, the effects of NT treatment in these single RW-challenged animals was so strong that we were unable to detect any difference in the production of Th2 cytokines between CON and NRW rats (not shown). Therefore, to amplify the cytokine expression in NRW-sensitized animals, we compared the responses of various groups after three RW challenges. The qPCR data presented in Fig. 6 show that RW treatment increased the lung mRNA expression of IL-4, IL-5, and IL-13 and that this expression was strongly down-regulated in NRW animals even after three i.t. RW challenges. Moreover, in the BAL the protein content of IL-4, and IL-13 was significantly reduced in NRW animals. Because the rat IL-25 ELISA kit is not currently available, lung sections from CON, RW, and NRW animals were examined by IHC for the expression of IL-25 protein. It is clear that the lungs from NRW animals had significantly lower IL-25 than the animals treated with RW alone (Fig. 8). Similarly, compared with RW-treated rats, the lung protein and mRNA expression of the eosinophil chemokine eotaxin, determined by IHC (Fig. 9A) and qPCR (Fig. 9B) analyses, respectively, was significantly reduced in NRW animals. These results suggest that NT strongly suppresses the allergen-induced Th2 cytokine/chemokine responses.
Nicotine does not decrease RW-induced mucous cell metaplasia or Muc5ac expression

Mucus hypersecretion is a key pathophysiological feature of human asthma. Because NT potently inhibits the expression of some key cytokines (e.g., IL-13 and IL-4) implicated in mucin production in the lung (45), we determined Muc5ac protein levels in the BAL using flexiVent plethysmography as described in Materials and Methods. Pooled data from three experiments using the peak resistance value for each dose of methacholine were used to plot the data (n = 4).

**FIGURE 12.** NT treatment does not decrease the airway resistance to methacholine. Rats were sensitized and challenged with RW as described in Fig. 1. Three days after the challenge, the airway resistance of CON, RW-sensitized/challenged, and NRW animals in response to increasing doses of methacholine was determined using flexiVent plethysmography as described in Materials and Methods. Pooled data from three experiments using the peak resistance value for each dose of methacholine were used to plot the data (n = 4).

**Discussion**

Increasing evidence suggests that smokers have lower incidences of allergic and lung inflammatory diseases (3, 4, 10, 11); however, the mechanism by which cigarette smoke modulates the allergic responses is not yet well understood. Allergic asthma is a Th2 disease and, in animal models of allergic asthma, airway inflammation is intimately associated with the increased production of Th2 cytokines and chemokines, particularly IL-13, IL-4, IL-5, and eotaxin (22, 23, 44). NT, a major constituent of cigarette smoke, suppresses innate and adaptive immune responses (2), but its effects on allergic responses are essentially unknown. The studies presented herein indicate that NT, which by itself has no significant effect on basal lung responses, dramatically suppresses the lung inflammation induced by two common human allergens, RW and HDM. All allergen-sensitized/challenged rats exhibited marked inflammation with abundant eosinophils in the lung; interestingly, however, even the few NT-treated animals (<25%) that developed mild but detectable leukocytic infiltration had very little eosinophilic infiltration in the lung. Eosinophils are highly related to allergic asthma in humans and animal models (48–50), and IL-5 and eotaxin are thought to be major cytokines/chemokines that enhance the differentiation, activation, expansion, mobilization, and in situ survival of eosinophils (24, 25, 49). Our results show that NT strongly suppresses eotaxin and essentially blocks IL-5 expression in the lung, and this inhibition might account for the near lack of eosinophilic infiltration in NT-treated animals in response to an allergen challenge.

In addition to IL-5, the other major cytokines produced during allergic asthma responses are IL-4, IL-13, and IL-25 (51). Both IL-4 and IL-13 signal through receptors containing IL-4Rα and cause airway inflammation and other symptoms of allergic asthma (51, 52). In mouse models of allergic asthma, IL-4 promotes Th2 responses and IL-13 is considered essential for AHR and goblet cell metaplasia. However, there is evidence that IL-4 promotes AHR and goblet cell metaplasia independently of IL-13 (53). Interestingly, NT essentially blocks the expression of both these cytokines in the lung without decreasing the allergen-induced increase in airway resistance to methacholine, goblet cell metaplasia, and mucus production. Indeed, compared with RW-sensitized/challenged animals, NRW animals exhibited a moderate increase in stored mucosubstances and in lung Muc5ac mRNA expression. Because NT decreased the Muc5ac protein content in the BAL, it is possible that the increased amount of mucosubstances within the goblet cells resulted from the decreased release of mucins from these cells. However, NT increased (albeit minimally) the expression of Muc5ac mRNA in the lung. Therefore, it is likely that mucous cell metaplasia in NRW animals resulted from both increased mucin synthesis and decreased mucin release from goblet cells. Given that NT strongly inhibits allergen-induced IL-4 and IL-13, the factor(s) that regulate increased Muc5ac mRNA expression and decreased secretion of mucosubstances in NT-treated animals are not clear. There is some evidence that IL-25 increases mucous cell metaplasia independently of IL-13 as well as in synergy with IL-13 (54). Therefore, it is possible that NT increased IL-25 in the lung and, in the presence of small amounts of IL-13, caused goblet cell metaplasia and increased mucin production. However, immunohistochemical examination of the lungs clearly indicated that NT down-regulates the expression of RW-induced IL-25 in the lung. Therefore, it is highly unlikely that NT modulates the goblet cell metaplasia and mucin production through IL-25.

The role of IL-13/IL-4 in the induction of mucin (e.g., Muc5ac) expression is not totally unequivocal. Some studies suggest not
only that IL-13 has no stimulatory effect but that it may actually inhibit mucin production (55–57). Therefore, it is likely that the effects of NT on mucous cell metaplasia are independent of its effects on IL-13/IL-4 synthesis. Other factors that might regulate the effects of NT on mucin production include the activation of epidermal growth factor receptor (58) and the excitatory γ-aminobutyric acid (GABA) receptor pathways (59). Although the contribution of these pathways to the modulation of airway allergic responses in NT-treated animals is currently unknown, strong interactions between nicotinic and GABA receptors exist in neuronal cells (60, 61); therefore, it is conceivable that NT modulates mucous production through GABA receptors.

CysteinyI leukotrienes, such as LTC₄, are potent bioactive lipids produced by a variety of cells, particularly mast cells, basophils, and eosinophils (62, 63). In human asthma, mast cells localize within the bronchial smooth muscle bundles and, upon degranulation, release histamine and LTC₄, causing bronchoconstriction (64). In comparison to histamine, LTC₄ causes ~1000 times more effective than histamine in contracting bronchial airway smooth muscles (63) and, therefore, critical in allergen-induced bronchoconstriction. As evidenced by the BAL content of hexosaminidase (data not shown), NT treatment does not affect the release of preformed granules but strongly suppresses the allergen-induced production of LTC₄ in the lung. Moreover, our preliminary studies indicate that NT treatment of the rat basophil cell line (RBL-2H3) also suppresses anti-FceRI-mediated LTC₄ synthesis without affecting hexosaminidase release (N. C. Mishra et al., unpublished data). Because bronchoconstriction in response to methacholine results from the interaction of methacholine with the muscarinic receptors on the bronchial smooth muscle cells, our results suggest that NT does not significantly affect the expression of muscarinic receptors on the airways. In addition to its bronchoconstricting property, LTC₄ is a strong chemotactic for eosinophils and neutrophils and promotes airway remodeling. LTC₄ receptor inhibitors (e.g., montelukast and zafirlukast) have demonstrated efficacy in attenuating eosinophil and neutrophil accumulation (65), smooth muscle hyperplasia, and airway remodeling (66). Mast cells also regulate airway remodeling by stimulating bronchial fibroblasts to produce collagen (67). Interestingly, NT inhibits the production of type I collagen by some cell types (68, 69). Thus, the leukotriene-inhibiting property of NT may contribute to the inhibition of the allergen-induced accumulation of eosinophils and other inflammatory cells in the lungs of NT-treated animals.

IgE provides the biological basis for allergy and immediate hypersensitivity. Recent evidence suggests that IgE plays a key role in the pathophysiology of asthma and contributes to the early and late phases of airway inflammation through its effects on mast cells (70). Unlike its effects on Th2 cytokines, NT treatment did not totally block the allergen-stimulated IgE production; however, it significantly decreased the total as well as allergen-specific IgE in the BAL and serum of the NT-treated animals. In contrast, NT did not significantly alter either the total or allergen-specific IgE in the lung or serum, nor did it significantly change the production of the RW-specific IgG subclasses, IgG1 and IgG2a. This is compatible with the observation that, unlike IgE, the production of IgG1, IgG2a, and IgG2b do not require IL-5 (71). Although the mechanism by which NT modulates IgE production in response to an allergen is not clear, given its differential effects on IgG and IgE, NT appears to inhibit the Ig class switch in response to allergens. IL-4/IFN-γ provides one critical signal for B cells to switch to IgE production (72), and the decreased IgE production in NT-treated animals might reflect the inhibitory effects of NT on IL-4/IFN-γ production. Therefore, in summary, the suppression of critical Th2 cytokines by NT might explain some beneficial effects of cigarette smoke on allergic asthma. However, cigarette smoke/NT is likely to exacerbate allergen-induced mucus production and unlikely to be protective against stress/irritant-induced AHR.

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