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Prenatal Secondhand Cigarette Smoke Promotes Th2 Polarization and Impairs Goblet Cell Differentiation and Airway Mucus Formation

Shashi P. Singh,* Sravanthi Gundavarapu,* Juan C. Peña-Philippides,* Jules Rir-sima-ah,* Neerad C. Mishra,* Julie A. Wilder,* Raymond J. Langley,* Kevin R. Smith, † and Mohan L. Sopori*

Parental, particularly maternal, smoking increases the risk for childhood allergic asthma and infection. Similarly, in a murine allergic asthma model, prenatal plus early postnatal exposure to secondhand cigarette smoke (SS) exacerbates airways hyperreactivity and Th2 responses in the lung. However, the mechanism and contribution of prenatal versus early postnatal SS exposure on allergic asthma remain unresolved. To identify the effects of prenatal and/or early postnatal SS on allergic asthma, BALB/c dams and their offspring were exposed gestationally and/or 8–10 wk postbirth to filtered air or SS. Prenatal, but not postnatal, SS strongly increased methacholine and allergen (Aspergillus)-induced airway resistance, Th2 cytokine levels, and atopy and activated the Th2-polarizing pathway GATA3/Lck/ERK1/2/STAT6. Either prenatal and/or early postnatal SS downregulated the Th1-specific transcription factor T-bet and, surprisingly, despite high levels of IL-4/IL-13, dramatically blocked the allergen-induced mucus cell metaplasia, airway mucus formation, and the expression of mucus-related genes/proteins: Muc5ac, γ-aminobutyric acid A receptors, and SAM pointed domain-containing Ets-like factor. Given that SS/nicotine exposure of normal adult mice promotes mucus formation, the results suggested that fetal and neonatal lung are highly sensitive to cigarette smoke. Thus, although the gestational SS promotes Th2 polarization/allergic asthma, it may also impair and/or delay the development of fetal and neonatal lung, affecting mucociliary clearance and Th1 responses. Together, this may explain the increased susceptibility of children from smoking parents to allergic asthma and childhood respiratory infections. The Journal of Immunology, 2011, 187: 4542–4552.
on the development of allergic asthma. To our knowledge, we show for the first time that gestational exposure to SS is by far the overwhelming risk factor for exacerbated AHR, Th2 polarization, and atopy and is associated with activation of GATA3/Lck/ERK1/2/STAT6. Surprisingly, unlike the promucoid effects of cigarette smoke/nicotine in adult humans and animals (27–29), both prenatal and early postnatal exposure to SS suppressed the various parameters of airway mucus response.

Materials and Methods

Animals

Pathogen-free BALbc mice from the Frederick Cancer Research Facility (Frederick, MD) were housed in shoebox-type plastic cages with hardwood chip bedding and conditioned to whole-body exposure in exposure chambers (H1000; Hazleton Systems) for 2 wk before exposure to SS for breeding (5). The chamber temperature was maintained at 26 ± 2°C, and lights were set to a 12-h on/off cycle. Food and water were provided ad libitum. The Animal Care and Use Committee of Lovelace Respiratory Research Institute approved all animal protocols.

Abs and reagents

All reagents, unless stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO). Cigarette smoke generation and exposure

Mice were exposed to whole-body SS (smoke released from the burning end of a cigarette) or filtered air (FA) for 6 h/d, 7 d/wk, as described (5). Briefly, a smoking machine (AMESA Type 1300; AMESA Technologies, Geneva, Switzerland) generated two 70-cm³ puffs/min from a research cigarette (type 2R1; Tobacco and Health Research Institute, Lexington, KY), and the smoke was captured from the lit end of the cigarettes with a plastic manifold placed above it. This level (total particulate matter, 1.52 ± 0.41 mg/m³) of exposure simulates the conditions at which a pregnant woman would be exposed to environmental tobacco smoke for 3 h/d in a smoking bar (5). Adult (3–4-mo-old) male and female mice were separately acclimatized to SS or FA for 2 wk prior to mating. After ascertaining pregnancy by vaginal smear, pregnant mice were housed singly in plastic cages and continued to receive SS or FA until the pups were born. The mothers and pups continued to be exposed to either FA or SS starting from day 1 of birth until the pups were weaned at 3 wk of age, and the pups continued to be exposed to FA or SS postnatally until sacrifice between 8–10 wk of age. This led to four groups of experimental animals, receiving the following combination of prenatal/postnatal exposure: FA/FA, FA/SS, SS/FA, and SS/SS.

Sensitization with allergen

The allergen used in the study was a lyophilized culture filtrate preparation of Aspergillus fumigatus; the filtrates (kindly provided by Dr. John M. Routes, Department of Pediatrics, Children’s Hospital, Wisconsin Medical College, Milwaukee, WI) were stored at −70°C until use. Mice were immunized intratracheally (i.t.) with A. fumigatus (50 µg/0.1 ml endotoxin-free sterile saline or sterile saline alone) and subsequently challenged i.t. with the A. fumigatus extracts (100 µg/0.1 ml) three times at 5-d intervals (5).

Total serum IgE

Total serum IgE levels were determined on diluted (1:100) serum using a mouse-specific serum IgE ELISA kit (MB Bioproducts, St. Paul, MN), according to the manufacturer’s instructions. The sensitivity of the assay was <2.0 ng/ml; the cross-reactivity with IgG was <0.01%.

Airway resistance

Forty-eight hours after the last A. fumigatus or saline challenge, airway resistance (R₂) was measured by the FlexiVent system (SCIREQ, Montreal, Quebec, Canada), as described (5). The peak R₂ response at the nebulized A. fumigatus (200 µg/ml) concentration and at each methacholine (MCh) concentration was used for data analysis.

Bronchoalveolar lavage fluid collection, cell differentials, and cytokine analysis

Established protocols were followed to obtain bronchoalveolar lavage fluid (BALF) from the animals (5). Briefly, mice were anesthetized and killed by exsanguination 48 h after the last A. fumigatus challenge. Before excision of the lungs, the trachea was surgically exposed, cannulated and, while the left lung lobe was tied off with a silk thread, the right lobe was lavaged twice with 1 ml sterile Ca²⁺/Mg²⁺-free PBS (pH 7.4). Aliquots were pooled from individual animals. Cell differentials and cytokine assays were performed, as described (5). Macrophage, neutrophil, lymphocyte, and eosinophil numbers were determined microscopically by counting ≥300 cells/sample. Lavage cytokines were assayed using the Mouse Cytokine Multiplex ELISA kit (Biosource-Invitrogen, Camarillo, CA), according to the manufacturer’s directions. The sensitivity of the assay was <10 pg/ml.

Immunohistochemistry

For immunohistochemical detection of airway mucus, formaldehyde-fixed left lung sections (5 µm) were stained with Alcian blue–periodic acid–Schiff (AB-PAS), as described previously (28). By this procedure, the mucus-producing cells stained distinctive pink and were examined microscopically at 40× magnification.

Analysis of mucosubstances in lung airways

The volume of mucous cells and the density of mucosubstances (VSD) in the airway epithelium were quantitated by a semiautomatic image-analysis system (28), using the public domain National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image.html). Morphometry was performed blinded, and the data were expressed as the mean ± SD VSD (nl/mm² basal lamina).

SAM pointed domain-containing Ets-like factor staining

Paraffin-embedded lung tissue (5 µm) was deparaffinized and rehydrated by placing slides on a slide warmer at 56°C for 20 min, followed by three 10-min incubations in xylene and a series of alcohol washes, followed by PBS. Ag retrieval was performed by immersing the slides in 10 mM citrate buffer at 90°C for 15 min. Endogenous peroxidase quenching was done by placing the slides in 3% H₂O₂ in methanol for 15 min at room temperature (RT), washing (five times in PBS), and blocking with 10% goat serum for 2 h at RT. The slides were incubated overnight at 4°C with a guinea pig anti-mouse polyclonal SAM pointed domain-containing Ets-like factor (SPDEF) Ab (GP954; a generous gift from Dr. Jeffrey Whitsett, Cincinnati Children’s Hospital, Cincinnati, OH) at 1:2500 dilution. Slides were washed five times with buffer and incubated at RT for 30 min with a 1:200 dilution of a goat anti-guinea pig biotinylated IgG (catalog no. BA-1000; Vector Lab, Burlington, CA). Slides were washed in PBS and incubated for 30 min in the Immunoperoxidase Kit (catalog no. PK-6100; Vector), per the manufacturer’s instructions. Slides were washed five times in buffer and incubated for 2 min at RT in peroxidase substrate (catalog no. SK-4423; Vector) without nickel. Slides were then counterstained with hematoxylin and dehydrated. Slides were examined at 40×; SPDEF stains brown.

Quantitative PCR

Total RNA was extracted from the frozen lung samples using TRI reagent (Molecular Research Center, Cincinnati, OH) and quantified per the manufacturer’s instructions. The lung expression of the airway mucin Muc5ac, the Th1 transcription factor T-bet, the Th2 transcription factor GATA3, and the housekeeping gene GAPDH was determined using primer/probe sets (Applied Biosystems, Foster City, CA). The relative expression (test mRNA/GAPDH) was calculated (5).

Western blot analysis

Lung tissues were homogenized in radioimmunoprecipitation assay buffer (20 mM Tris, 150 mM NaCl, 20 mM β-glycerophosphate, 1% Triton X-100, 10 mM NaF, 5 mM EDTA, 1 mM Na₃VO₄) containing protease inhibitors (1 mM PMSF; 1 µg/ml each aprotinin, antipain, and leupeptin) at 4°C. Protein content and Western blot (WB) analysis were performed, as described (5). NF-κB (p65), GATA3, STAT5, STAT6, and Lck were determined by probing the blots with anti–phospho-NF-κB-p65 (Ser²⁷, rabbit polyclonal; Abcam, San Francisco, CA), anti–phospho-GATA3 (Ser³⁰¹, rabbit polyclonal; Abcam), anti–phospho-STAT5 (Tyρ³⁰⁴/³⁰⁷, rabbit polyclonal; Cell Signaling Technology, Danvers, MA), and anti–phospho-Lck (Tyρ³⁰⁴, rabbit polyclonal; Cell Signaling Technology). The γ-aminobutyric acid A receptor (GABAAR) and T-bet expression was determined by probing the blot with an anti–GABAAR (mouse monoclonal; Milipore, Temecula, CA) and anti–T-bet Ab (mouse monoclonal; Abcam). Blots were developed with ECL (Amersham Biosciences, U.K.) using x-ray photograph film.
Results

Prenatal, but not postnatal, exposure to SS increases AHR

We previously showed that prenatal plus perinatal exposure to SS increased R_l to MCh (5); however, individual contributions of prenatal and postnatal SS exposure on AHR were not addressed. Therefore, dams were exposed to SS or FA, as described in Materials and Methods; 17 d prior to sacrifice, four groups of animals (FA/FA, FA/SS, SS/FA, and SS/SS) were sensitized with A. fumigatus. R_l was determined in response to aerosolized MCh (Fig. 1A) or a predetermined optimal concentration of aerosolized A. fumigatus (200 μg/ml) (Fig. 1B). Results demonstrated that, compared with FA/FA, both MCh and A. fumigatus caused dramatic increases in the R_l in prenatally SS-exposed animal groups (SS/SS and SS/FA); however, the R_l values between FA/FA and FA/SS groups and between SS/SS and SS/FA groups were not significantly different, indicating that, unlike prenatal SS exposure, postnatal SS exposure does not affect R_l significantly. MCh-induced changes in R_l values of mice not sensitized to A. fumigatus indicated that, even in the absence of allergen, prenatally SS-exposed mice (SS/FA and SS/SS) were significantly more sensitive to MCh than FA/FA or FA/SS mice (Supplemental Fig. 1). Even the basal R_l of prenatally SS-exposed mice was somewhat higher (statistically significant) than that of FA/FA or FA/SS mice. These results suggested that, although prenatal SS exposure strongly exacerbates MCh- and A. fumigatus-induced AHR, SS exposure during the early postnatal life had a relatively minor effect on this response. Moreover, in this model, changes in AHR reached the level that allowed R_l measurements in response to the allergen (A. fumigatus). R_l measurements in non-A. fumigatus-sensitized mice indicated that prenatal exposure to SS exacerbated MCh-induced AHR; however, the degree of the response was considerably lower than in A. fumigatus-sensitized mice (Supplemental Fig. 1). Thus, prenatal SS exposure primes the lung for exacerbated AHR response.

Prenatal SS exposure induces a strong Th2 cytokine response in the lung

The Th2 cytokines, particularly IL-13 and IL-4, play an essential role in allergic asthma and Th2 polarization (30, 31); however, only AHR responses, and not Th2 responses, are ameliorated by the phosphodiesterase4-selective inhibitor rolipram. This suggested that AHR and Th2 responses are regulated by different mechanisms (5) and, therefore, it was possible that prenatal and early postnatal SS exposure might affect AHR and Th2 polarization differently. To ascertain this possibility, we determined the A. fumigatus-induced IL-4 and IL-13 production in four groups of mice: FA/FA, SS/FA, FA/SS, and SS/SS. Results presented in Figure 1C and 1D indicated that, compared with control mice (FA/FA), the early postnatal exposure to smoke (FA/SS) produced a relatively small, but significant, increase in the BALF levels of the Th2 cytokines IL-4 (Fig. 1C) and IL-13 (Fig. 1D) in response to A. fumigatus sensitization. However, these levels were much lower than those present in the BALF from prenatally SS-exposed animals (IL-4: 13.48 ± 3.74 and 6.30 ± 1.92, SS/FA and FA/SS, respectively; IL-13: 111.7 ± 19.82 and 34.58 ± 15.65 ng/ml, SS/FA and FA/SS, respectively). Compared with A. fumigatus-sensitized mice, the levels of IL-4 and IL-13 in nonsensitized mice were very low (Supplemental Fig. 2). These results suggested that prenatal exposure to SS is the predominant risk factor in driving the lung toward Th2 polarization. In contrast, the levels of IL-4,

**FIGURE 1.** Prenatal, but not postnatal, exposure to SS exacerbates airway reactivity, strongly upregulates allergen-induced Th2 cytokine expression, and increases total serum IgE levels. Lung resistance (R_l; cm H_2O s/ml) was measured using FlexiVent system in response to increasing doses of computer-controlled nebulized MCh (A) and a single nebulized dose (200 μg/ml) of A. fumigatus (B). Inset in A shows baseline differences between the groups in response to nebulized saline. Peak responses at each MCh concentration and response to A. fumigatus were used for data analysis. IL-4 (C) and IL-13 (D) levels were determined in BALF from A. fumigatus-sensitized mice by the Multiplex ELISA kit. E. Total IgE levels in the serum of A. fumigatus-sensitized mice were quantitated by using a mouse-specific IgE ELISA kit. All animals were sensitized with A. fumigatus, as described in Materials and Methods prior to R_l determination. The results are representative of animal responses from two different sets of inhalation exposures. Data are expressed as mean ± SD (n = 5–7/group). *p ≤ 0.05, ***p ≤ 0.001.
and IL-13 in mice without *A. fumigatus* challenges, did not reach statistical significance, indicating that the critical levels of these cytokines are achieved only after allergic sensitization.

**Prenatal exposure to SS increases leukocytic infiltration in the lung after A. fumigatus sensitization**

Elevated numbers of eosinophils and neutrophils in the lung are associated with allergic asthma (5). To determine whether pre- and/or postnatal exposure to SS differentially affected the leukocytic infiltration in the lung in response to an allergic challenge, *A. fumigatus*-sensitized FA/FA, FA/SS, SS/FA, and SS/SS mice were challenged i.t. with *A. fumigatus* extracts. The volume of BALF recovered was not significantly different among the groups. BALF cells were collected, cytospun, and stained to obtain the differential cell count. Prior to *A. fumigatus* sensitization, the total number of leukocytes in the BALF in both FA and SS groups was similar (6.8 ± 2.6 × 10^6; however, after *A. fumigatus* challenge (+), the number of cells in FA/FA* and FA/SS* increased to 51–54 ± 8.3 × 10^6. The number increased to 92–98 ± 12.8 × 10^6 in SS/FA* and SS/SS* animals. Thus, *A. fumigatus* promoted leukocytic infiltration in the lungs, and the infiltration was significantly more pronounced in the animals exposed to SS prenatally; postnatal SS did not result in a significant difference in the total number of cells in the BALF (Table I).

The differential BALF cell count (Table I) indicated that macrophages were the predominant cell population in the BALF from FA/FA, FA/FA*, and FA/SS*, accounting for 90.8 ± 4.4, 95 ± 1.6, and 92.1 ± 2.6%, respectively. Thus, although the *A. fumigatus* challenge strongly increased the total number of BALF cells, the percentages of macrophages did not vary significantly among the groups; nonetheless, animals exposed postnatally to SS (FA/SS) had small, but significantly higher, numbers of neutrophils. In contrast, prenatally SS-exposed groups (SS/FA and SS/SS) showed a dramatic decrease in the proportion of macrophages, with concomitant increases in neutrophils and eosinophils. These results suggested that prenatal exposure strongly primes the lung for neutrophilic and eosinophilic inflammation.

**Prenatal SS increases total serum IgE levels**

Atopy is a strong risk factor for allergic asthma in children (32). To ascertain whether prenatal and/or postnatal SS exposure affects atopy differentially, we measured the serum levels of IgE in the four groups of mice after *A. fumigatus* sensitization. Results (Fig. 1E) indicated that compared with FA/FA, prenatal (SS/FA) and early postnatal (FA/SS) exposure independently elevated total serum IgE levels; however, the IgE level in SS/FA (prenatally SS-exposed animals) of 29.50 ± 14.11 µg/ml was significantly higher than the IgE level of 8.81 ± 2.49 µg/ml in FA/SS (SS exposure only during the early postnatal period). Although, the mean IgE concentration of 39.83 ± 2.75 µg/ml in SS/SS (prenatally and postnatally SS exposure) was higher than in prenatally SS-exposed animals, it did not reach statistical significance. These results suggested that although prenatal and early postnatal exposure is independent risk factors for atopy, prenatal exposure is a substantially stronger risk factor.

**Prenatal SS activates GATA3, ERK1/2, LCK, and STAT6**

Th2 cytokines are intimately associated with allergic asthma, and coincident with Th2 development is activation of the IL-4/STAT6 pathway that enhances the expression of GATA3 (33). GATA3 is the master transcription factor for Th2 differentiation and, under physiological conditions, it is selectively expressed in Th2 but not Th1 cells and induces Th2 cytokine gene expression (33, 34). Lck, ERK, and STAT6 regulate GATA3 activity (34–36). Therefore, we examined the lung expression of GATA3, Lck, ERK, and STAT6 by quantitative PCR and/or WB analysis. Fig. 2A shows a representative response of various groups of SS-exposed mice. Mice exposed gestationally to SS (SS/FA and SS/SS) and subsequently exposed to *A. fumigatus* exhibited significantly higher levels of activated (phosphorylated) GATA3. Increased GATA3 expression in prenatally SS-exposed animals was also seen by quantitative PCR analysis (Fig. 2B). Moreover, prenatal, but not postnatal, exposure to SS activated phosphorylated ERK1/2; total ERK1/2 was not affected by either pre- or postnatal SS (Fig. 2C). Similarly, prenatal, but not postnatal SS, increased activation (phosphorylation) of Lck (Fig. 3A) and STAT6 (Fig. 3B); neither prenatal nor postnatal SS affected the expression of actin (Fig. 3C). These results suggested that prenatal, but not postnatal SS activates the Th2-signaling pathway (GATA3/Lck/ERK/STAT6). *A. fumigatus* sensitization is critical for the visualization of SS-induced exaggerated Th2 and AHR responses; without *A. fumigatus* sensitization, the baseline phosphorylation of GATA3/STAT6 (Supplemental Fig. 3) and Lck/ERK1/2 (data not shown) was comparable between various groups. Thus, activation of GATA3/Lck/ERK/STAT6 is associated with an exacerbated allergen-induced asthmatic phenotype following gestational exposure to SS.

**Prenatal SS does not affect allergen-induced NF-κB and STAT5 activation**

NF-κB, a ubiquitously expressed transcription factor plays a vital role in inflammatory responses and is activated in some chronic lung diseases, such as COPD (37). NF-κB has also been implicated in IL-13–induced lung pathology (38). Serine phosphorylation of NF-κB p65 subunit was shown to be important in the function of NF-κB as a transcription factor (39); therefore, we determined the level of phosphorylated NF-κB p65 in the lung extracts as an index of NF-κB activation. As seen in Fig. 4A, although allergic sensitization increased phosphorylated NF-κB p65, neither prenatal nor postnatal SS exposure significantly affected the magnitude of this activation; therefore, it is unlikely

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**Table I. Leukocytic composition of BALF cells**

<table>
<thead>
<tr>
<th>Leukocyte Subtype</th>
<th>FA/FA (Pre- and Postnatal FA)</th>
<th>FA/FA* (Pre- and Postnatal FA) + A. fumigatus Extract</th>
<th>FA/SS* (Postnatal SS) + A. fumigatus Extract</th>
<th>SS/FA* (Prenatal SS) + A. fumigatus Extract</th>
<th>SS/SS* (Pre- and Postnatal SS) + A. fumigatus Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total BALF leukocytes</td>
<td>6.2 ± 2.6 × 10^6</td>
<td>51.4 ± 8.3 × 10^6</td>
<td>54.0 ± 6.9 × 10^6</td>
<td>92.4 ± 11.3 × 10^6</td>
<td>97.8 ± 12.6 × 10^6</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>90.8 ± 4.1</td>
<td>95 ± 1.58</td>
<td>92.1 ± 2.65</td>
<td>31.6 ± 4.18*</td>
<td>38.0 ± 5.54*</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>3.8 ± 2.06</td>
<td>4.5 ± 1.48</td>
<td>11.5 ± 2.11</td>
<td>35.0 ± 4.62*</td>
<td>38.1 ± 6.46*</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.7 ± 0.60</td>
<td>0.2 ± 0.24</td>
<td>2.1 ± 0.53</td>
<td>1.9 ± 0.33*</td>
<td>2.0 ± 0.37*</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0 ± 0.00</td>
<td>0.3 ± 0.24</td>
<td>1.3 ± 0.34</td>
<td>34.3 ± 4.02*</td>
<td>36.6 ± 5.11*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (n = 5–6).

*Not significant compared with FA/FA* and FA/SS*.

*p < 0.05 compared with FA/FA* and FA/SS*.
that exacerbated allergic asthma associated with prenatal SS exposure is linked to activation of NF-κB. Early production of the Th2 cytokine IL-4 is regulated by phosphorylation of the transcription factor STAT5 (40); however, results presented in Fig. 4B showed that neither pre- nor postnatal SS significantly altered activation (phosphorylation) of STAT5. Together, these results suggested that NF-κB and STAT5 are not strongly associated with the SS-induced exacerbated Th2 responses. Prenatal and early postnatal SS exposure strongly suppresses the allergen-induced T-bet expression in the lung GATA3 and T-bet are two opposing transcription factors for Th1 and Th2 development, respectively (40, 41). Results (Fig. 5) clearly showed that sensitization of control (FA/FA) animals with *A. fumigatus* (FA/FA*) strongly increased the level of T-bet in the lung; however, animals exposed to SS either prenatally (SS/FA) or postnatally (FA/SS) exhibited a dramatic reduction in the *A. fumigatus*-induced T-bet protein levels (Fig. 5A). WB data presented in Supplemental Fig. 3 (top panel) show that exposure to an allergen is essential to upregulate T-bet expression. Quantitative PCR data indicating that prenatal and early postnatal exposure to SS downregulated the expression of T-bet mRNA (Fig. 5B) also supported these results. Thus, prenatal and early postnatal exposure to SS suppresses the allergen-induced T-bet expression, which might impair the development of Th1 responses in the lung. To evaluate the effect of SS exposure on the prototype Th1 cytokine, IFN-γ levels were assayed in the BALF of the experimental animals. Although the various groups did not exhibit significant differences in the basal (nonsensitized) levels of IFN-γ (Supplemental Fig. 2C), FA/FA animals exhibited a significant increase in the BALF levels of IFN-γ after *A. fumigatus* sensitization. In contrast, the IFN-γ levels in animals exposed either prenatally and/or postnatally to SS failed to upregulate the BALF levels of IFN-γ after the *A. fumigatus* challenge (Fig. 5C). Thus, SS exposure affected T-bet and IFN-γ similarly.

**Prenatal and/or early postnatal exposure to SS suppresses mucus formation and expression of Muc5ac and GABAARs in the lung**

Mucus produced by mucosal epithelial cells in the respiratory tract acts as the first line of defense against inhaled pathogens, which are cleared by the mucociliary apparatus (42, 43). However, dysregulated mucus formation is an important factor in many lung diseases, including asthma. Recent evidence suggests that GABAARs and IL-13 are intimately associated with the airway mucin Muc5ac and mucus formation by bronchial epithelial cells (44, 45). To ascertain the effects of prenatal and postnatal SS exposure on mucus formation, we determined the expression of Muc5ac (Fig. 6A) and GABAARs (Fig. 6B) by quantitative PCR and WB analysis, respectively, as well as the presence of mucus in the lung (Fig. 7) by immunohistochemistry. As expected, in control (FA/FA) animals, *A. fumigatus* sensitization led to significant increases in the airway mucus content (Fig. 7A, 7B). Surprisingly, however, the amount of mucus was dramatically reduced in the airways during early postnatal (Fig. 7D), prenatal (Fig. 7C), and prenatal plus postnatal (Fig. 7E) SS exposure. The histopathologic data were validated by determining the Vc of mucousubstances in the mucosal surface epithelium of the lung airways (Fig. 7F). These results were further supported by the observation that prenatal and
postnatal SS exposure independently decreased the expression of Muc5ac (Fig. 6A) and GABAARs (Fig. 6B) in the lung. Without allergic (A. fumigatus) challenge, GABAAR expression is similar in all groups (Supplemental Fig. 3, panel 4). Thus, prenatal and/or early postnatal SS exposure suppresses mucus production that might adversely impact the mucociliary clearance and defense against inhaled pathogens/noxious particulate agents in these animals.

**Prenatal exposure to SS suppresses SPDEF expression in the lung airways**

SPDEF is the transcription factor that plays an important role in the development and differentiation of pulmonary goblet cell and mucus production (46). Because SPDEF is primarily restricted to goblet cells, we ascertained the expression of SPDEF in airway epithelial cells by immunohistochemistry. Results presented in Fig 7 show that SPDEF is expressed moderately in nonsensitized FA/FA airways (Fig. 7G); upon sensitization with A. fumigatus, FA/FA animals strongly upregulated the expression of SPDEF (Fig. 7H). However, A. fumigatus sensitization of prenatally SS-exposed animals (SS/FA) failed to increase the expression of SPDEF in the airways; indeed, the expression of SPDEF in SS/FA animals (Fig. 7I) was even lower than the basal levels in control FA/FA animals. Drastic reduction in SPDEF levels was also seen in FA/SS animals (data not shown). Thus, the inability of allergens to upregulate the expression of SPDEF in prenatally and/or early postnatally SS-exposed animals may be the main cause for

**FIGURE 3.** Prenatal SS activates Lck and STAT6. Lung homogenates (70 μg) were analyzed by WB. A, The blots were probed with anti phospho (p)-Lck Ab. Right panel, Densitometry analysis of blots from three separate experiments. B, The blots were probed with anti p-STAT6 Ab. Right panel, Densitometry analysis of blots from three separate experiments. C, The blots were probed with anti-β-actin Ab. The figures are representative of three separate analyses. Bars represent the mean ± SD. ***p ≤ 0.001.

**FIGURE 4.** SS does not affect NF-κB or STAT5 activation. Lung homogenates (75 μg protein) were analyzed by WB, and the blots were probed with anti-phosphorylated-p65 Ab (A) or anti–phosphorylated–STAT5 Ab (B). Data are representative of three separate sets of analyses. A and B, Right panels, Densitometry analysis of blots from three separate experiments. Bars represent the mean ± SD. ***p ≤ 0.001.
impaired differentiation of goblet cells and mucus production in these animals.

**Discussion**

Th2 bias, atopy, and AHR are the hallmarks of allergic asthma (3–5, 47) and, in animal models, prenatal plus perinatal exposure to mainstream or secondhand cigarette smoke increases AHR and Th2 lung inflammation (25); however, AHR and Th2 polarization do not overlap mechanistically (5). Therefore, it is possible that prenatal and perinatal exposure to cigarette smoke affect AHR and Th2 lung inflammation differentially. In this article, we showed that gestational, but not early postnatal, exposure to SS primes the lung to dramatic increases in allergen-induced airway resistance ($R_L$) as well as Th2 inflammation (increased IL-4 and IL-13 levels) and increased levels of serum IgE. Although the allergen sensitization of gestationally SS-exposed animal caused a significant increase in the serum IgE levels, the IgE may or may not be specific to the allergen. A mild increase in AHR has been reported after early postnatal exposure to SS (48), these studies used Penh values to quantitate AHR. However, the value of Penh in deter-

**FIGURE 5.** Prenatal and/or postnatal SS suppresses T-bet and IFN-$\gamma$. A, Representative results of lung homogenates (75 $\mu$g protein) analysis by WB, and probed with anti–T-bet Ab. Right panel, Densitometry analysis of blots from three independent experiments. B, T-bet mRNA expression in the lung tissue analyzed by quantitative PCR, as described in Materials and Methods. C, IFN-$\gamma$ level in BALF, as described in Materials and Methods. ($n = 5$/group.) Bars represent the mean $\pm$ SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

**FIGURE 6.** SS exposure suppresses Muc5ac and GABA$\alpha_R$ expression. A, Muc5ac mRNA expression in the lung tissue analyzed by quantitative PCR ($n = 5$/group). B, Representative result of three independent WB analyses of lung homogenate (75 $\mu$g protein) probed with anti-GABA$\alpha_R$ Ab. Right panel, Densitometry analysis of blots from three separate experiments. Bars represent the mean $\pm$ SD. ***$p \leq 0.001$. 
mining lung function and airway resistance has been strongly challenged (49, 50). Therefore, there is no tangible evidence that early postnatal exposure to SS increases airway resistance significantly.

The mechanism by which cigarette smoke exacerbates Th2 responses is largely unknown. Activation of GATA3 is intimately associated with Th2 polarization and suppression of Th1 responses; on the contrary, T-bet promotes Th1 and suppresses Th2 polarization (51–54). We observed that although A. fumigatus sensitization in normal (FA/FA) animals resulted in an upregulated expression of both GATA3 and T-bet, mice exposed prenatally to SS exhibited a strong upregulated expression of GATA3 and a dramatic suppression of T-bet, indicating while gestational SS promotes Th2 polarization, it simultaneously downregulates Th1 activation. Effects of SS exposure on the Th1 cytokine IFN-γ were similar to those seen with T-bet; thus, exposure to SS causes parallel changes in T-bet and IFN-γ. Although prenatal and/or early postnatal exposure to SS suppressed T-bet, effects of early postnatal SS on GATA3 expression were only weakly higher than control. Thus, although both prenatal and early postnatal SS exposure downregulate T-bet and IFN-γ, increased GATA3 expression is primarily associated with prenatal SS exposure. GATA3 is known to decrease T-bet expression (54); however, it is not clear whether GATA3 is the only factor that downregulates T-bet in gestationally SS-exposed animals. Nonetheless, given that Th1 responses are important in clearing infections, decreased T-bet could increase the risk for protracted lung infections and, at least in part, explain the increased risk for infections among children exposed to cigarette smoke through parental smoking (55–57).

Among the factors that promote Th2 development is the IL-4–STAT6 pathway that enhances the expression of GATA3 and Th2 polarization (34). Although STAT5 has also been implicated in Th2 responses, this transcription factor primarily affects the early IL-4 production (40), and the A. fumigatus-induced activation of STAT5 was not significantly affected by either prenatal or postnatal SS exposures. In contrast, although prenatal exposure strongly activated STAT6, activation of STAT6 by postnatal SS was much weaker. Thus, activation of STAT6 may play a significant role in activation of GATA3 by prenatal SS exposure. In addition to STAT6, a number of other factors promote GATA3 activation during Th2 polarization. Lck controls GATA3 and IL-4 production, and Lck−/− Th2 cells have lower levels of IL-4 and GATA3 and a higher expression of T-bet and IFN-γ (36). Moreover, activated ERK inhibits ubiquitination and degradation of GATA3 in Th2 cells (35), thereby increasing the level of activated GATA3. In contrast, activated Lck and ERK negatively regulate T-bet expression (35, 36). Our results showed that Lck and ERK1/2 are activated in the lungs of prenatally SS-exposed animals, and these factors are likely to contribute to the increased GATA3 and decreased T-bet expression. The observation that early postnatal SS only weakly activated Lck/ERK1/2/GATA3 and Th2 polarization also supported a potential correlation between stronger upregulated activation of Lck/ERK1/2/GATA3 and stronger Th2

FIGURE 7. SS exposure suppresses the allergen-induced airway mucus and SPDEF formation. Lung sections (5 μm) were stained with AB-PAS for mucus and SPDEF, as described in the Materials and Methods. Representative histologic photomicrographs (40×) of the lung sections show the mucus-producing cells in pink and SPDEF in brown. Mucus staining: FA/FA (A), FA/FA+ (B), FA/SS+ (C), SS/FA+ (D), and SS/SS+ (E). F. Volume density (Vv) of AB-PAS–stained mucous substances in the mucosal surface in the lung epithelium (n = 5/group). Bars represent the mean ± SD. ***p ≤ 0.001. SPDEF staining: FA/FA (G); FA/FA+ (H), and SS/FA+ (I) (n = 3/group).
polarization in gestationally SS-exposed animals. However, although it is likely that STAT6 and Lck/ERK strongly trigger Th2 polarization in prenatally SS-exposed animals, other potential mechanisms of GATA3 activation contributing to Th2 polarization in these animals cannot be ruled out. For example, cigarette smoke was shown to elevate lung inflammation through epigenetic changes involving chromatin modifications (58), and pro-inflammatory cytokines, such as IL-6 (59, 60) also activate GATA3.

NF-κB is the transcription factor that regulates proinflammatory cytokine production, and it may play an important role in IL-13–induced lung pathogenesis (38). Activated NF-κB contains NF-κBp50 and phosphorylated NF-κBp65 subunits (61), and our results indicated that although A. fumigatus sensitization causes a sharp increase in p65, neither prenatal nor early postnatal SS significantly altered the magnitude of this response. Thus, although NF-κB might be important in allergic sensitization, it is unlikely to play a critical role in SS-induced exacerbated allergic asthma.

Direct methods, such as MCh or histamine, are commonly used to assess AHR; however, this approach has several drawbacks (62). It was observed that increased AHR detected by direct stimuli, such as histamine or MCh, is not specific to allergic asthma and may be associated with a number of other lung diseases, such as COPD, sarcoidosis, and bronchiectasis. Moreover, a significant number of normal subjects may also show increased AHR to these agents (reviewed in Ref. 63). In contrast, indirect stimuli (e.g., allergens) act on cell types, such as inflammatory and neuronal cells, to transmit the signal to effector cells and are considered a better indicator of asthma and inflammation (63). However, in general, allergen-specific AHR changes are much weaker than MCh-induced AHR changes and, therefore, are difficult to quantify. To our knowledge, allergen-induced AHR has not been reported in animal models, although the effects of allergens on isolated lungs and tracheal rings have been described (64, 65). Given the magnitude of MCh-induced Rl in prenatally SS-exposed animals, we determined whether the prenatal SS exposure was a sufficiently strong trigger for a significant increase in allergen-induced Rl. Indeed, prenatal, but not postnatal, SS exposure strongly increased the A. fumigatus-induced Rl, indicating that prenatal SS exposure is a strong stimulus for allergic asthma and an excellent animal model to test the efficacy of interventions for allergic asthma-associated AHR.

Mucus production is a cardinal feature of bronchial asthma and is associated with goblet cell metaplasia (66). IL-13 and IL-4 play a critical role in mucus formation (45, 67); however, despite large increases in Th2 cytokines, including IL-13/IL-4 in gestationally SS-exposed animals, prenatal and early postnatal SS exposure dramatically reduced airway mucus formation. Although IL-13 may induce lung inflammation and Muc5ac through the ERK1/2/MAPK pathway, independent of STAT6 (68, 69), STAT6 also controls Th2 cytokines and goblet cell metaplasia (70). Thus, despite the presence of IL-13 and activated STAT6 and ERK1/2, prenatal and early postnatal SS exposure suppresses goblet cell formation and mucus production. This observation was counterintuitive, because cigarette smoke is a strong promucus stimulus in humans and adult mice (71). Muc5ac is the major inductive mucin in the airways, its expression is controlled by GABAARs in airway epithelial cells (72), and the expression of both Muc5ac and GABAARs was strongly downregulated by pre- and/or early postnatal exposure to SS. Mammalian lung development starts in the fetus and continues for a significant period after birth (i.e., several weeks to several years in mice and humans, respectively) (73). It is possible that SS exposure during this critical period blocks or delays development of type II airway epithelial cells. The transcription factor SPDEF plays an important role in the growth and differentiation of goblet cells, and our results suggested that the lung expression of this critical transcription factor is downregulated by pre- and/or early postnatal SS exposure. Together, these results suggested that SS affects the differentiation of airway epithelial cells into goblet cells. Recently, Fu et al. (72) reported more GABAAR-expressing cells in the pulmonary neuroepithelial bodies from monkeys exposed gestationally to nicotine, suggesting an increased potential for mucus formation. A likely explanation for this is that cigarette smoke is a very complex mixture of thousands of chemicals, and some of these chemicals might affect early lung development/maturatation. Prenatal and early postnatal exposure to SS might at least temporarily impair the developmental process and make cells either hypo- or hyperresponsive to various growth and differentiation factors. Indeed, prenatal nicotine was reported to adversely affect cellular communication and normal lung development (74), and early postnatal SS exposure impaired Clara cell secretary protein levels (15). We have some preliminary evidence that gestational exposure to SS affects lung development and the development of type II cells (S.P. Singh and M.L. Sopori, unpublished observations). Thus, prenatal and/or early postnatal SS exposure may impair/delay the development/differentiation of airway goblet cells and reduce mucus production, even in the presence of high levels of IL-13/IL-4 and activated STAT6 and ERK1/2.

The mucociliary apparatus is important in the clearance of pathogens from the respiratory tract, and mucosal epithelial cells act as the first line of defense against respiratory pathogens (42). Although excessive mucus production may contribute to the morbidity of some respiratory diseases, diminished mucus formation is likely to encourage respiratory infections. Together with suboptimal Th1 development through decreased T-bet, loss of mucus formation may increase the susceptibility and length of respiratory infections, as well as explain the increased risk for respiratory infections among children from mothers who smoke cigarettes. Overall, these studies strongly suggested that a fetus is exceptionally sensitive to cigarette smoke, which may promote the development of childhood allergic asthma and respiratory infections, and every effort should be made to dissuade women from being exposed to cigarette smoke, including environmental tobacco smoke, during pregnancy.

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References


Supplementary FIGURE S1. Airway resistance ($R_L$) in the absence of allergic (Af)-challenge. $R_L$ were measured using FlexiVent system on mice (without Af challenge) in response to nebulized saline (0 mg/mL MCh), and MCh. The results are representative of animal responses from two different sets of inhalation exposures. Animal groups are the same as described in the figure legend for Figure 1 in the main text. FA = filtered air, SS = secondhand cigarette smoke; FA/FA (prenatal FA/postnatal FA); FA/SS (prenatal FA/postnatal SS); SS/FA (prenatal SS/postnatal FA); SS/SS (prenatal SS/postnatal SS). Data are expressed as mean ± SD (n = 5). ** p < 0.01, *** p < 0.001, and NS = not significant.
Supplementary Figure S2. IL-4, and IL-13 in the BALF from the mice without allergic (Af) challenge. IL-4 (A), IL-13 (B), and INF-γ (C) levels were measured in the BALF from unimmunized mice by Multiplex ELISA kit (Biosource-Invitrogen, Camarillo, CA) according to the manufacturer’s directions. Differences in the cytokines level are not statistically significant without allergic challenge between the exposure groups. FA = filtered air; SS = secondhand cigarette smoke; FA/FA (prenatal FA/postnatal FA); FA/SS (prenatal FA/postnatal SS); SS/FA (prenatal SS/postnatal FA); SS/SS (prenatal SS/postnatal SS). Data are expressed as mean ± SD (n = 5-7). NS = statistically not significant.
Supplementary FIGURE S3. Absence of upregulation of p-STAT6, p-GATA3, T-bet, and GABA\textsubscript{A}R without allergic (Af) challenge. Western blot analysis of lung homogenates (70 µg protein) of mice exposed to secondhand cigarette smoke without Af-challenge. Blots were probed with the respective antibody indicated in the Online Data Supplement: Material and Method. Representative blot of T-bet (probed with anti-T-bet antibody, mouse monoclonal, Abcam), phospho (p)-STAT6 (probed with anti-p-STAT6 (Tyr\textsuperscript{641}, rabbit polyclonal; Cell Signaling Tech. Inc., MA), GATA3 (probed with anti-p-GATA3 (Ser\textsuperscript{308}, rabbit polyclonal; Abcam), and GABA\textsubscript{A}R (probed with anti-GABA\textsubscript{A}R, mouse monoclonal, Millipore, CA), are shown in the figure above (n=3). FA = filtered air. SS = secondhand cigarette smoke; FA/FA (prenatal FA/postnatal FA); FA/SS (prenatal FA/postnatal SS); SS/FA (prenatal SS/postnatal FA); SS/SS (prenatal SS/postnatal SS). FA/FA+ (lane 5, the last lane) shows allergic challenge (+ = Af-challenged mice) is required to up-regulate T-bet and GABA\textsubscript{A}R.