Cigarette smoke suppresses Th1 cytokine production and increases RSV expression in a neonatal model

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Phaybouth, Vatsana, Shan-Ze Wang, Julie A. Hutt, Jacob D. McDonald, Kevin S. Harrod, and Edward G. Barrett. Cigarette smoke suppresses Th1 cytokine production and increases RSV expression in a neonatal model. Am J Physiol Lung Cell Mol Physiol 290:L222–L231, 2006. First published August 26, 2005; doi:10.1152/ajplung.00148.2005.—Respiratory syncytial virus (RSV) infects ~90% of young children by the age of 2 yr, with peak rates occurring during 2–6 mo of age. Exposure to side-stream cigarette smoke (SS) may increase the incidence or manifestation of an RSV infection. We hypothesized that exposure to SS would alter the subsequent immune response to RSV infection in neonatal mice. BALB/c mice were exposed to air or 1.5 mg/m³ of SS from day (d) 1 up to 35 d of age. A subset was intranasally infected with 4 x 10⁴ PFU of RSV/g body wt on d 7 and rechallenged at 28 d of age. Immune responses were assessed on d 4 and 7 after RSV rechallenge. Both air- and SS-exposed mice responded to SS rechallenge with neutrophilia and decreased Clara cell secretory protein levels within the lung. However, an increase in bronchoalveolar lavage fluid eosinophils, in addition to reduced levels of Th1 cytokines (IFN-γ and IL-12), decreased lung tissue inflammation, and decreased mucus production was observed in SS-exposed mice compared with air-exposed mice after RSV rechallenge. Ultimately changes in cytokine and inflammatory responses due to SS exposure likely contributed to increased viral gene expression. These results suggest that SS exposure plays a significant role in shaping the neonatal response to RSV infection.

 Worldwide, respiratory syncytial virus (RSV) is the leading cause of infant mortality from respiratory infections. RSV is so highly contagious that by age 2 yr almost all children have been infected (13) with peak rates between 2 and 6 mo of age (17). Recovery from primary infection does not result in complete immunity; thus RSV regularly infects children and adults, causing common colds, exacerbating preexisting lung disease, and contributing to winter respiratory morbidity and mortality in the elderly (9). Severe infantile RSV infections are associated with recurrent wheezing and asthma diagnosis in later life (40). Currently, there is no effective therapy for RSV infection due, in part, to the poorly understood pathogenesis of RSV-induced airway disease, but lung-specific host defense appears to be important in modulating lung pathogenesis during infection (25). Therefore, factors that effect host defense may be important determinants of the outcome of RSV pathogenesis and future lung disease.

Exposure to parental cigarette smoking, especially that of the mother, has been identified as one of the major risk factors for respiratory infections in children (32). It is estimated that 50% of children under the age of 5 yr are exposed to environmental tobacco smoke (ETS, or secondhand smoke) after birth (22). ETS is composed of exhaled mainstream and side-stream cigarette smoke (SS; the components that are emitted into the air during the burning of a tobacco product). Previous studies have shown SS to act as an adjuvant on eosinophils, allergenspecific antibodies, and Th2 cytokines [interleukin (IL)-4 and IL-10] in adult mice previously sensitized by injection with ovalbumin (OVA) and aluminum hydroxide (37). In addition, SS can exacerbate existing asthmatic symptoms in humans and animal models of disease (37, 38). The factors or mechanisms associated with SS exposure that may predispose an individual to increased lung problems are still unclear.

In addition to the impact of environmental pollutants on respiratory infections, the maturity of the immune system at the time of infection plays a significant role in mediating the response to infection. A recent study demonstrated in a BALB/c mouse model that timing of the first RSV infection determines the polarity of RSV-specific immunological memory that develops and, in turn, influences the severity and nature of disease following subsequent reinfections (9). Culley et al. (9) proposed that animals initially infected during early infancy generate a Th2-polarized RSV-specific immunological memory that upon reinfection as adults is expressed in the form of intense infiltrates of eosinophils and IL-4-secreting CD4+ Th2 cells in the lung. In the current study we developed a neonatal murine model to examine the role of SS exposure in modulating the host immune response to primary neonatal RSV infection followed by a secondary infection later in life. The goal of this study was to investigate whether SS exposure will alter the immune response to subsequent RSV infections, thereby indicating an influence of SS exposure on the neonatal response and ability to affect the severity of disease outcome.

MATERIALS AND METHODS

Animals. Wild-type BALB/c pregnant female mice were purchased from Charles River Laboratories (Wilmington, MA) and housed under pathogen-free housing conditions according to the Association for Assessment and Accreditation of Laboratory Animal Care-approved guidelines and protocols. Mice arrived on day 14 of their 19- to 21-day gestation period. Newborn mice were housed in solid-bottom cages and were weaned at day 21 of age. Mothers and their offspring were exposed to SS or filtered air and offspring were subsequently weaned at day 21 of age.

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infected with RSV or vehicle (VEH) according to the schedule depicted in Fig. 1. Although offspring were killed after both primary (days 2, 4, and 7) and secondary (days 4 and 7) VEH treatment or RSV infection, the primary focus of the study is on the response after secondary infection due to the minimal response observed following primary infection. All animal procedures for this study were reviewed and approved by the Institutional Animal Care and Use Committee of Lovelace Respiratory Research Institute.

**RSV.** A stock of RSV A2 strain was propagated in Hep-2 cell cultures, and viral titration was performed as described previously (15). Briefly purified RSV was titrated in triplicate for each culture dilution by standard plaque assay procedures. Plaque formation was counted manually by visualization following hematoxylin staining of cell monolayers.

**Mouse model of RSV infection.** On day 7 after birth neonatal mice were given a 10-μl inoculate containing either 4 × 10⁴ plaque-forming units (PFU) of RSV per gram body weight or (RPMI) by intranasal instillation (average body wt 15 g). Secondary infection occurred on day 28 of age by intranasal instillation of a 50-μl inoculate containing either 4 × 10⁴ PFU of RSV per gram body weight or RPMI (average body wt 15 g) while the animals were lightly anaesthetized.

**Smoke exposure.** One day after birth, newborn mice (mother and litter) were placed in whole body exposure chambers and exposed to either air or SS (target concentration = 1.5 mg/m³). Neonatal mice were exposed to SS for 7 days/wk, 6 h/day until killed. Generation of SS has been described previously (2). In brief, SS exposure conditions were established by smoking machines (type 1300; AMESA Electronics, Geneva, Switzerland) and by capturing the smoke from the lit end of the cigarette (14RF; Kentucky Tobacco Research and Development Center, Lexington, KY). A plastic manifold was placed above the cigarettes, and the smoke was diluted with filtered air and delivered to the exposure chambers. The mass concentration of SS total particulate material (TPM) was determined by gravimetric analysis of filter samples taken every 2 h during the exposure period. Other mice were housed in the same room in chambers that received filtered air (Air) only. Separate chambers were provided for RSV-infected animals.

**Exposure atmosphere characterization.** Table 1 reports the average and standard deviations among all daily particulate matter (PM) measurements in the infected and noninfected animal exposure chambers. Additionally, carbon monoxide (CO), total hydrocarbon (THC), nicotine/cotinine, and cigarette smoke particle size distribution were measured in the noninfected exposure chamber. PM concentration was measured gravimetrically after sampling for 30-min intervals on 47-mm glass fiber (Pall-Gelman, East Hills, NY) filters. CO and THC concentrations were determined with a Photoacoustic Gas Analyzer (Innova 1312; California Analytical Instruments, Irvine, CA). The vendor performed the initial calibration, and calibration was checked on-site against National Institute of Standards and Technology traceable gases. Particle size was measured using a combination of a scanning mobility particle sizer (SMPS) (model 3080; TSI, St. Paul, MN) for particle number size distribution and a multijet Mercer cascade impactor (5) for PM size distribution. The SMPS was operated at a sample flow rate of 0.3 l/min and a sheath flow rate of 3 l/min. Particles were counted from the SMPS exit on a condensation particle counter (model 3025, TSI). SMPS data were analyzed with Aerosol Instrument Manager (version 5.0, TSI).

To capture both the gaseous and particle-bound portion of nicotine and cotinine they were collected on vapor-sorbing impregnated filters (SIFs). SIFs were made with glass fiber filters (47 mm Pallflex, Pall-Gelman) impregnated with finely ground XAD-4 sorbent (Sigma-Aldrich). After sampling (two filters in series to ensure 100% capture of analytes), we stored coated filters at −20°C before extraction. Samples were spiked with deuterated nicotine and cotinine internal standards and then extracted by microwave-assisted solvent extraction (MARS-X; CEM, Houston, TX) in ultrapure dichloromethane. Extracts were evaporated to ~50 μl under gentle nitrogen stream and brought to 1 ml with acetonitrile before analysis by gas chromatography/mass spectrometry (HP 5890 GC/5972 MS).

**Pathologic analysis.** Mice were killed, on days 2, 4, and 7 after primary infection and days 4 and 7 after secondary infection, by injection with a lethal dose of a pentobarbital-based euthanasia solution. We obtained bronchoalveolar lavage (BAL) cells by inserting a catheter into the trachea and lavaging the lung three times with 0.5 ml for neonates or 0.8 ml of phosphate-buffered saline (PBS, without calcium chloride and magnesium chloride). Total BAL cells were determined with a hemacytometer. BAL cells were spun onto slides by cytocentrifugation and stained with a modified Wright-Giemsa stain. Two hundred counts were determined, and the percentage of specific cell types was determined for each animal.

Separate mice were killed on day 7 after primary and secondary infection and were used for histopathology analysis. Lungs were infused via the trachea with 10% neutral buffered formalin, the trachea was ligated, and lungs were immersed in formalin. The lungs were removed and fixed in the same solution. After paraffin embed-

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**Table 1. Particle size and content of cigarette smoke exposure atmosphere**

<table>
<thead>
<tr>
<th>Particle size</th>
<th>Sham (LOQ)</th>
<th>Cigarette Smoke (LOQ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass median aerodynamic diameter (μm ± GSD)</td>
<td>&lt;LOQ</td>
<td>0.2 ± 0.2 ± 0.2</td>
</tr>
<tr>
<td>Number median diameter (nm ± GSD)</td>
<td>&lt;LOQ</td>
<td>130 ± 30</td>
</tr>
</tbody>
</table>

**Gases**

| Nitrogen monoxide (ppm) | <LOQ | 0.23 ± 0.08 ± 0.02 |
| Nitrogen dioxide (ppm) | <LOQ | <LOQ ± 0.02 |
| Carbon monoxide (ppm) | 0.1 ± 0.1 ± 0.01 |
| Total vapor hydrocarbon (ppm) | 0.1 ± 0.5 ± 0.05 |
| Nicotine and cotinine (μg/m³) | <LOQ | 143 ± 14 ± 14 |
| Cotinine (μg/m³) | <LOQ | 10 ± 2 ± 2 |

Measurements were obtained from noninfected animal chamber only. Values are means ± SD. *Measurement in parenthesis obtained from infected animal chamber. †Geometric standard deviation. ‡Less than limit of quantitation (LOQ).
ing, 5-μm-thick sections for microscopic analysis were stained with hematoxylin, eosin, and Alcian blue. Lung lesions for each animal, including alveolar septal infiltrates, perivascular infiltrates, combined bronchus-associated lymphoid tissue hyperplasia, and peribronchiolar infiltrates were subjectively graded on a severity scale of minimal, mild, moderate, and marked [corresponding to numbers 1 (minimal) to 4 (marked)]. Individual lesion scores were summed from each animal to create an overall histopathology score for each animal (2). A pathologist who was blinded to the exposure conditions evaluated all slides.

**RT-PCR analysis of viral gene expression.** Total RNA was isolated from the lobes of the right lung using TRI-reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer’s protocol. RT-PCR analysis was designed to detect nascent viral mRNA transcripts but not detect genomic or progeny RSV RNA, as a measure of viral transcriptional activity as described previously (20). Virus-specific mRNA transcripts for the RSV-F gene were converted to cDNA by the reverse transcription reaction using the following virus-specific primer sequences: RSV-F, 5'-CAACTCCATTGTTATTCC-3', RSV-Specific sequences were amplified by PCR using the following primer sets: RSV-F Upper Primer, 5'-CCAGCAAAGTGTAGACCTAAAAA-3'; RSV-F Lower Primer, 5'-AATGCCACCGTTAGAAATGCT-3'. Primer sequences were identified with Lasergene software (DNAStar, Madison, WI). The RSV-F-specific sequences were amplified for 30 cycles, and RT-PCR amplification products were visualized by ethidium bromide-stained gel electrophoresis under UV light illumination. Gel images were captured, and densitometric analysis was performed with the GelDoc documentation system and Quantity One software (Bio-Rad, Hercules, CA). RT-PCR analysis of endogenous β-actin mRNA steady-state levels was used as loading and assay controls.

**Inflammatory cytokine analysis.** The left lung lobe was homogenized in 1 ml of PBS and centrifuged at 1,200 rpm for 7 min at 4°C. The supernatants were collected for analysis of cytokine protein levels. The levels of IL-5, IL-12, IL-13, and IFN-γ were measured using enzyme-linked immunosorbent assay kits and following the manufacturer’s protocol (OptEIA; Pharmingen, San Diego, CA). 

**Immunohistochemistry analysis.** For immunohistochemical studies, lung sections were prepared as described previously (21). In brief, lung sections (5 μm) were taken at equivalent distances from the histological reference point and were stained overnight with primary rabbit antibodies (a generous gift from Barry Stripp, University of Pittsburgh) against Clara cell secretory protein (CCSP). Slides were rinsed in physiological buffer and incubated with secondary goat anti-rabbit antibodies conjugated to biotin (Vector Laboratory, Burlingame, CA). A streptavidin-conjugated peroxidase detection system (Vector Laboratory) was used to visualize antibody-binding complexes following incubation with diaminobenzidine. Multiple sections from each tissue block were analyzed under light microscopy. CCSP staining was quantified by adaptation of mucus cell metaplasia (MCM) staining procedure as described previously (18). For MCM quantification, lung sections were stained with Alcian blue and periodic acid-Schiff (AB/PAS) as described previously (39). For quantification of MCM, intrapulmonary bronchi were analyzed for the volume of mucusubstances by Scion Image software (National Institutes of Health, Bethesda, MD) and mucusubstance calculations normalized to mm² of basal lamina as described previously (18).

**Statistical analysis.** Data are expressed as means ± SE. For data sets of normal distribution, two-way analysis of variance (ANOVA) with Bonferroni posttest was performed to determine main effects of and interactions between RSV infection and SS exposure. When interactions were significant, one-way ANOVA with Bonferroni posttest was used to determine significance between groups. For nonnormal distributed data sets (cytokine data), statistical analysis using logarithmic transformation of data to normal distribution sets and subsequent two-way or one-way ANOVA was performed. T-test was performed when only two groups were being compared. A value of \( P < 0.05 \) was considered significant. Data analysis was performed with GraphPad Prism version 4.0 software (GraphPad Software, San Diego, CA).

**RESULTS**

**Viral gene expression.** The RSV-F gene was expressed readily in the lungs of the Air/RSV mice (Fig. 2A) and barely detectable in the SS/RSV mice (Fig. 2B) 2 days following primary RSV infection. On days 4 and 7 following primary RSV infection, RSV-F gene expression was not detectable in the lungs of either the Air or SS exposure group (data not shown). In contrast RSV-F gene expression was readily detectable in both Air/RSV and SS/RSV groups 4 days following secondary RSV infection (Fig. 2C). Densitometric analyses of RSV-F mRNA levels indicate a significant increase in RSV-F expression in the lungs of SS/RSV mice compared with Air/RSV mice 4 days following secondary infection (Fig. 2D). On day 7 postsecondary infection, the RSV-F gene was no longer detectable in either Air/RSV or SS/RSV groups (data not shown). β-Actin mRNA steady-state levels did not change in the lungs of either smoke-exposed mice or air-exposed mice at any time following RSV infection.

**Lung inflammation.** To determine the effect of SS exposure and RSV infection on lung inflammation, bronchoalveolar lavage fluid (BALF) cellularity from SS-exposed and Air-
exposed mice were assessed at 4 and 7 days following secondary RSV infection. The number of neutrophils was significantly increased in the BALF of both Air- and SS-exposed mice following RSV infection (Table 2). SS exposure did not independently affect neutrophil numbers in the BALF. Eosinophil numbers were significantly increased in SS/RSV-exposed mice 4 days following RSV infection; however, there was no increase in Air/RSV-exposed mice (Table 2). Lymphocyte and macrophage population(s) did not significantly differ between groups (data not shown). Primary RSV infection did not induce any significant changes in inflammatory cell numbers (data not shown). Total BALF cell numbers were significantly increased only in SS/RSV-exposed mice 4 days following RSV infection compared with SS/VEH-exposed mice or Air/RSV-exposed mice (data not shown).

To further determine the role of SS exposure and RSV infection in modulating lung inflammatory responses, we analyzed lung inflammation and epithelial morphology by histological staining of lung sections. Lung sections from Air/VEH-exposed mice 7 days following primary or secondary treatment did not exhibit any discernable pathology (Fig. 3: A primary, B secondary). Secondary infection with RSV resulted in a significant increase in the mean histopathological score in both Air/RSV- and SS/RSV-exposed animals (Table 3). The pathology was characterized by marked mucus cell metaplasia, which tended to involve the larger airways and distal bronchioles, and increased peribronchial-perivascular inflammation, which included alveolar macrophages and/or neutrophilic infiltrates (Fig. 3D). Interestingly, the histopathological score for the SS/RSV-exposed group was significantly less than the Air/RSV-exposed group.

Table 2. Inflammatory cell populations in BALF collected 4 and 7 days following secondary vehicle or RSV challenge in Air- or SS-exposed mice

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Neutrophil × 10⁵/ml</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Neutrophil × 10⁵/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air/VEH</td>
<td>0.053 ± 0.020</td>
<td>0.105 ± 0.091</td>
<td>4</td>
<td>5</td>
<td>0.107 ± 0.038</td>
<td>0.126 ± 0.063</td>
</tr>
<tr>
<td>Air/RSV</td>
<td>0.017 ± 0.017</td>
<td>1.740 ± 1.360</td>
<td>4</td>
<td>4</td>
<td>3.055 ± 1.529</td>
<td>1.098 ± 0.341‡</td>
</tr>
<tr>
<td>SS/VEH</td>
<td>0.058 ± 0.019</td>
<td>0.234 ± 0.076</td>
<td>4</td>
<td>6</td>
<td>0.100 ± 0.035</td>
<td>0.136 ± 0.043</td>
</tr>
<tr>
<td>SS/RSV</td>
<td>6.022 ± 1.620*</td>
<td>0.744 ± 0.197</td>
<td>4</td>
<td>6</td>
<td>3.848 ± 1.217†</td>
<td>1.578 ± 0.380‡</td>
</tr>
</tbody>
</table>

Values represent means ± SE of each group. BALF, bronchoalveolar lavage fluid; RSV, respiratory syncytial virus; SS, side-stream cigarette smoke; VEH, vehicle. *P < 0.01 vs. Air/VEH, Air/RSV, and SS/VEH on day 4; †P < 0.05 vs. Air/VEH and SS/VEH on day 4; ‡P < 0.005 vs. SS/VEH and Air/VEH on day 7; §P < 0.05 vs. Air/VEH and SS/VEH on day 7.

Fig. 3. Lung tissue inflammation 7 d following secondary RSV infection is decreased in the lungs of SS-exposed mice compared with air-exposed mice after secondary RSV infection. Hematoxylin and eosin-stained lung sections (5 μm) were assessed by light microscopy, and lung histopathology and inflammation were blindly scored on a 0–4 point scale. Photomicrographs (magnification at ×150) were taken from representative slides of all exposure groups 7 d following primary or secondary VEH treatment or RSV infection: Air/VEH (A), Air/VEH (B), Air/RSV (C), Air/RSV (D), SS/VEH (E), SS/VEH (F), SS/RSV (G), SS/RSV (H).
Table 3. Histopathological scoring of lung tissue inflammation 7 days following secondary vehicle treatment or RSV infection in air- or SS-exposed animals

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>Mean Pathological Score</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air/VEH</td>
<td>0.400 ± 0.400</td>
<td>5</td>
</tr>
<tr>
<td>Air/RSV</td>
<td>3.800 ± 2.000*</td>
<td>5</td>
</tr>
<tr>
<td>SS/VEH</td>
<td>0.200 ± 0.200</td>
<td>5</td>
</tr>
<tr>
<td>SS/RSV</td>
<td>2.000 ± 0.578**</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P ≤ 0.05 vs. Air/VEH, SS/VEH, and SS/RSV; †P ≤ 0.05 vs. Air/VEH, Air/RSV, and SS/VEH.

RSV-exposed group (Table 3). There were no discernible histopathological differences between mice killed after primary VEH treatment or RSV infection following either air or SS exposure.

Inflammatory mediators. The levels of IL-5, IL-12, IL-13, and IFN-γ were assessed in the lung homogenates of SS- and air-exposed mice at 4 and 7 days following either VEH treatment or secondary RSV infection. SS exposure alone led to a significant decrease in IL-12 and IFN-γ levels at both the 4- and 7-day time points following VEH treatment (Fig. 4). RSV infection alone did not have an effect on IL-12 or IFN-γ levels. Although IFN-γ levels remained suppressed in SS-exposed mice even after RSV infection IL-12 levels increased following RSV infection (SS/RSV). However, IL-12 levels in SS/RSV-exposed mice on day 7 postinfection were still significantly less than levels in Air/RSV-exposed mice. IL-5 and IL-13 protein levels in the lung homogenates were similar amongst all exposure (Air or SS) and/or treatment (VEH or RSV) groups (i.e., no effect of SS and/or RSV; data not shown).

Mucus cell metaplasia. The role of SS in modulating RSV-induced mucus production was examined by AB/PAS staining of the airways. Minimal mucus staining was visible in the airway epithelium of both Air- and SS-exposed mice following primary and secondary VEH treatment (Fig. 5 A, B, E, and F; Table 4). Both primary and secondary infection with RSV in air-exposed mice (Air/RSV) led to a significant increase in the number of cells staining positive for mucus, 32.270 ± 8.397 and 78.410 ± 12.99 cells/mm, respectively (Fig. 5, C and D; Table 4). Numbers of mucus cells in secondary infected mice were significantly higher than primary infected mice (P < 0.05). Although increased size of mucosubstances was observed in SS-exposed and RSV-infected mice (primary and secondary), it was only present within intermittent regions of the airways (e.g., not statistically significant; Fig. 5, G and H). Furthermore, the number of cells staining positive for mucus in SS/RSV-exposed animals was significantly less than Air/RSV-exposed animals (secondary infection only; Table 4).

CCSP production in airway epithelium. To assess other airway epithelial functions that may be altered during RSV infection by exposure to SS, CCSP was assessed by immunohistochemical staining of lung sections. There were no effects of SS and/or RSV exposure at 7 days postprimary infection on the intensity of CCSP staining in the airways (Fig. 6, A, C, E, and G; quantitative results not shown). However, following secondary RSV infection alone there was a significant decrease in CCSP staining (Fig. 6D, Table 5). Additionally, CCSP levels were significantly decreased in mice only exposed to SS and killed 7 days postsecondary VEH treatment (Fig. 6F, Table 5). Combined SS exposure and secondary RSV infection led to a significant reduction in CCSP staining that was equivalent to the levels following SS or RSV exposure alone (Fig. 6H, Table 5).

DISCUSSION

A neonatal murine model was developed to better understand the effects of RSV infection in infants on the development of immune function and maturation of the lungs. Furthermore, this neonatal model was used to examine the role of SS exposure on the immune response to a subsequent primary and secondary RSV infection. The immune response to primary RSV infection was relatively mild in both air- and smoke-exposed mice. Secondary RSV infection alone elicited a stronger immune response that included viral persistence, a significant influx of neutrophils, increased mucus cell numbers, and decreased CCSP levels within their lungs. Mice exposed to
SS alone for a period of 35 days (e.g., postsecondary treatment) had reduced IL-12, IFN-γ, and CCSP levels within the lungs. In combination, SS exposure had several effects on the immune response to secondary RSV infection, including increased viral persistence, increased number of eosinophils in the BALF, decreased lung tissue inflammation, and decreased numbers of mucus cells in the airways. Levels of IL-12 and IFN-γ remained suppressed following SS/RSV exposure suggesting these cytokines may have contributed to the increase in viral load and BALF eosinophilia present after secondary RSV infection. These findings suggest that SS cigarette smoke may play a role in modulating lung inflammatory and immune responses to RSV infections in newborns.

Epidemiological and experimental evidence shows a strong association between exposure to cigarette smoke in utero and/or postnatally and decreased lung function (4). Separating the independent effects of in utero vs. postnatal SS exposure is difficult because mothers who smoke will usually smoke during and after pregnancy. There seems to be an independent effect of postnatal SS exposure, as reports have shown an increase in hospital admissions for respiratory illnesses in the first 18 mo of life due to paternal smoking (7), which also led to decreased pulmonary function in older children 8–16 yr of age (7). Furthermore, the severity of RSV bronchiolitis early in life is modified by postnatal maternal cigarette smoke exposure and age of the infant (3). Therefore, the effects of postnatal

Table 4. Quantitative analysis of mucus cell staining in the lungs 7 days following secondary vehicle treatment or RSV infection in air- or SS-exposed animals

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>Number of Mucus-stained Cells, cells/mm of basal lamina</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air/VEH</td>
<td>3.366±3.293</td>
<td>5</td>
</tr>
<tr>
<td>Air/RSV</td>
<td>78.410±12.99*</td>
<td>5</td>
</tr>
<tr>
<td>SS/VEH</td>
<td>0.165±0.165</td>
<td>5</td>
</tr>
<tr>
<td>SS/RSV</td>
<td>19.930±12.97</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P ≤ 0.05 vs. Air/VEH, SS/VEH, and SS/RSV.
exposure to SS on a subsequent RSV infection in a neonatal model was investigated.

The concentration of SS used in this study (1.5 mg/m³ TPM) is just inside the high end (2 mg/m³ TPM) of the range to which humans are exposed in ambient settings (11, 12). However, infants and young children may actually be exposed to much higher concentrations than those normally reported in homes, as they are directly affected by the source, mothers who smoke. For example, higher urinary cotinine concentrations are found in children from smoking mothers than from smoking fathers (8). In addition, urinary cotinine amounts are also higher in bottle-fed infants from smoking mothers than in adults exposed to SS (36). Exposures in the current study were whole body exposures, thus newborns were likely exposed to SS components via multiple routes including, inhalation, dermal, maternal (breast milk and fur), and bedding. Exposure to SS factors via maternal and bedding exposure likely continued after the end of SS generation on any given day. Passive exposure to smoke is associated with measurable nicotine levels in breast milk (10). The contribution of each of these individual exposure routes with respect to the newborn’s responses is not known.

Table 5. Quantitative analysis of CCSP staining in the lungs 7 days following secondary vehicle treatment or RSV infection in air- or SS-exposed animals

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>Volume Density of Staining, nl/mm² of basal lamina</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air/VEH</td>
<td>1.093 ± 0.238</td>
<td>5</td>
</tr>
<tr>
<td>Air/RSV</td>
<td>0.336 ± 0.114†</td>
<td>5</td>
</tr>
<tr>
<td>SS/VEH</td>
<td>0.524 ± 0.103*†</td>
<td>5</td>
</tr>
<tr>
<td>SS/RSV</td>
<td>0.360 ± 0.120†</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are means ± SE. CCSP, Clara cell secretory protein. *P ≤ 0.05 vs. Air/VEH; †P ≤ 0.01 vs. Air/VEH.

The effect of exposure to SS during primary and secondary RSV infection on a Th1 vs. Th2 cytokine response was investigated. Exposure to SS suppressed both IL-12 and IFN-γ
production in RSV-infected and surprisingly even in VEH-treated groups. Cigarette smokers have reduced numbers of Th1 cytokine-secreting cells in their airways (16). Secondary RSV infection in SS-exposed mice did induce a significant increase in IL-12 levels although they remained significantly below the levels in RSV only (Air/RSV)-infected mice. This may be because some Th1 response to RSV infection was induced that was stronger than the suppressive effects of SS exposure. No significant effect of SS or RSV exposure was observed for the Th2 cytokines, IL-5 and IL-13, in lung tissue following primary or secondary infection. In a previous study mice infected with RSV at 1 or 7 days of age and rechallenged at 12 wk of age developed a Th2 biased response characterized by an increase in IL-4+ CD4+ and a decrease in IFN-γ+ CD4+ lymphocytes in the lungs (9). Differences in the timing of infection and the maturational state of the animal may explain the differences between studies. Herein, primary RSV infection was given to 7-day-old mice and reinfection occurred at 4 wk of age, whereas the previous study infected mice at day 1 or 7 and reinfefted them at 12 wk of age. Additionally, because viral inoculate depended on body weight (4 × 10^4 PFU of RSV per g body wt) mice at 4 wk of age received less viral inoculate than mice at 12 wk of age, which may have affected the outcome of response.

An imbalance in Th1/Th2 cytokines, with deficient Th1 and excess Th2 responses to RSV infection, has been demonstrated to be associated with impaired viral clearance (24). However, a skewed Th2 response may still develop in the absence of an increase in Th2 cytokines when there is a selective downregulation of Th1 cytokines such as IFN-γ (24). Children whose RSV-induced bronchiolitis was so severe as to require hospitalization exhibit significantly reduced levels of IFN-γ and increased levels of eosinophils (33, 35). Therefore, there might not only be an increased Th2 like inflammation in subjects who suffer severe RSV-induced bronchiolitis but also a decreased Th1 response (35). The present findings suggest that exposure to SS may bias the immune system toward a Th2 response by suppressing Th1 responses, which are necessary for viral clearance.

Viral persistence reflects a balance between viral replication and viral clearance that can alter the severity or duration of disease. Herein, RSV persistence was demonstrated as RSV-F gene expression. RSV-F gene expression was detectable only on day 2 after primary infection but was also detectable on day 4 after secondary infection for both air- and smoke-exposed mice. In addition, RSV-F gene expression was increased in the lungs of smoke-exposed mice 4 days after secondary infection compared with air-exposed mice. Previous studies indicate that exacerbation of lung disease by RSV infection can induce expression of other viral genes, such as RSV-G, suggesting that increased viral gene expression is not confined to a single viral gene (20). It has been shown previously that impairment of respiratory immune defense can be induced by chronic mainstream tobacco smoke exposure (34). Therefore, the observed increase in lung viral gene expression in SS-exposed mice may result from increased persistence of the initial viral inoculate or diminished antiviral innate mechanisms of viral clearance in the lung. The increase in viral gene expression observed in smoke-exposed mice may also be the result of a suppressed IL-12 and IFN-γ levels. The host molecular determinants that regulate RSV infection and replication have not been fully elucidated at the molecular level in in vivo settings.

SS exposure and subsequent RSV infections altered the outcome of lung tissue inflammation, which did not correlate with BALF eosinophil influx. Lung tissue inflammation in SS-exposed mice 7 days after secondary RSV infection was significantly higher than smoke-exposed VEH-treated mice. However, lung tissue inflammation in SS-exposed mice 7 days after secondary infection was significantly lower than air-exposed mice 7 days after secondary RSV infection. In contrast, there was a significant influx of eosinophils into the airways of SS-exposed mice 4 days after secondary RSV infection compared with air-exposed mice. Because no animals were killed for characterization of tissue inflammation (e.g., histopathology endpoints) at 4 days postinfection it is unknown whether tissue inflammation would have correlated with the inflammatory cells in the BALF at that time point. It is unclear why a significant influx of eosinophils occurred in SS-exposed mice but not in air-exposed RSV-infected mice, as previous studies have shown that rechallenge with RSV leads to eosinophilia that is associated with a biased Th2 response (9). In contrast, secondary infection with RSV led to an increase in BALF neutrophils; however, this was not further altered by SS exposure. Chronic exposure of newborn mice to SS alone does not lead to a significant increase in lung inflammation (2). Whether the altered severity of tissue inflammation and influx of eosinophil is associated with reduced ratio of Th1/Th2 cytokines in SS-exposed animals is not clear. However, it is possible that this altered response may have an effect on the ability to control viral replication.

In accordance with the increase in tissue inflammation, MCM increased in air-exposed mice after secondary RSV infection compared with VEH-treated and smoke-exposed mice. The increase in mucus-positive cells after RSV infection may be the result of increased recruitment of neutrophils to the lung. Neutrophils are the predominant leukocytes in the Airways of infants with RSV-induced bronchiolitis (14, 31, 41), and recruitment into the lungs can contribute to mucus production through release of neutrophil elastase (1, 23). Both smoke-exposed and air-exposed mice had a significant increase in neutrophil numbers 4 and 7 days after secondary RSV infection compared with VEH controls. This is consistent with previous studies showing increased neutrophilia in mice rechallenged with RSV (9).

It is unclear why neutrophil recruitment to the lungs of RSV-infected mice exposed to SS was not associated with an increase but rather a significant decrease in mucus production. Previous studies have shown that CXCR2, a chemokine receptor, induced by RSV infection, functions by increasing mucus production, which, when treated with anti-CXCR2, inhibits mucus production without differences in neutrophil influx (27). Therefore, it is possible that SS functions through this pathway, thereby leading to a decrease in mucus, but has no effect on neutrophil recruitment. Additionally, mainstream smoke exposure (~3 wk) attenuates OVA-induced airway inflammation in OVA-sensitized mice (26). However, these effects are thought to be mediated by high smoke-induced carboxyhemoglobin (HbCO) levels, as short-term mainstream smoke exposure that is associated with lower levels of HbCO actually exacerbates OVA-induced allergic airway responses in mice (26, 28). The levels of HbCO were not measured in the present study.
To assess other airway epithelial functions that may be modulated in the murine model, we assessed CCSP, believed to play a role in regulating inflammatory and immune responses to infection or injury (19, 43), by immunohistochemical staining of lung sections. CCSP can inhibit the chemotaxis and phagocytosis of neutrophils and monocytes, respectively (29, 30). CCSP-deficient mice exhibit increased viral persistence, increased lung inflammation, and increased Th2 cytokines and neutrophil chemokines in the lung following primary RSV infection (42). In our study, exposure of mice to SS alone for 35 days (e.g., postsecondary treatment) caused CCSP to decrease. A similar reduction in CCSP levels in the airways was seen following secondary RSV infection. However, there was no further reduction in mice exposed to SS and infected with RSV. Short-term exposure to high levels of aged and diluted cigarette smoke (6 h/day, 3 days, 10 mg/m³ PM) when combined with ozone (24 h, 0.5 ppm) exposure is associated with a decrease in CCSP levels and increased lung inflammation (44). Interestingly, the same exposures in IL-6-deficient mice prevents the loss of CCSP expression. High-level diesel engine emission (DEE) exposure (1,000 μg/m³ PM) alone has been shown to reduce CCSP staining in terminal airways. Moreover, low (30 μg/m³ PM) and high levels of DEE exposure also led to reduced CCSP staining in the airway epithelium following RSV infection (20). Therefore, CCSP expression appears to be a common target/mediator in response to many environmental insults. In previous studies, RSV infection alone had little effect on CCSP (20), whereas in our study secondary RSV infection of air-exposed mice showed a decrease in CCSP compared with primary infection or VEH treatment. The decrease in CCSP following RSV infection observed in our study may be the result of primary infection during the neonatal period, thereby affecting the level of CCSP when reinfection with RSV occurs later in life.

This study presents novel findings regarding the impact of SS exposure on pulmonary responses to a common viral pathogen in a neonatal murine model. Primary RSV infection alone during the neonatal period may prime the lungs to respond more vigorously to subsequent infection(s) (9). Furthermore, postnatal SS-induced alterations in IL-12 and IFN-γ cytokine levels are associated with altered RSV viral replication and pathogenesis of the infection. SS and RSV exposure may act synergistically to influence the inflammatory and immune responses that underlie the pathological processes in infection and other respiratory diseases, such as asthma. In conclusion, avoiding SS exposure and/or early RSV infection(s) may possibly reduce the severity or frequency of respiratory symptoms that may appear in later life.

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GRANTS

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