

## Exposure to Sidestream Tobacco Smoke Induces Airway Hyperresponsiveness and Inflammation in Early Postnatal Mice

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### Abstract

There are abundant epidemiological studies linking early environmental tobacco smoke (ETS) exposure with childhood asthma. The present study was designed to determine changes on airway responsiveness and innervation after ETS exposure during early postnatal period. The postnatal day (PD) 2 mice were exposed to either ETS or filtered air (FA) for 10 consecutive days. Lung function, substance P (SP) airway innervation, nerve growth factor (NGF) and cytokines in Bronchoalveolar Lavage (BAL) fluid were measured 1, 3 and 7 days after last ETS exposure. 1 day after the last ETS exposure, airway responsiveness and SP innervation in airway smooth muscle were significantly increased, as well as altered airway inflammation, which include significant elevated levels of IL-1 $\beta$ , IL-4, IL-6, IL-10, TNF $\alpha$  and INF $\gamma$  in BAL compared with FA exposure. Interestingly, 3 days after ETS exposure, airway hyperresponsiveness and SP innervation in airway smooth muscle remained elevated, parallel with high levels of IL-1 and NGF. These results indicated that the change in SP airway innervation play an important roles in airway hyperresponsiveness induced by ETS exposure during early in life.

**Keywords:** Airway innervation; Asthma; Neurokinin receptor; Substance P

**Abbreviations:** AHR: Airways Hyperresponsiveness; BALF: Bronchoalveolar Lavage Fluid; C<sub>dyn</sub>: Dynamic Pulmonary Compliance; ELISA: Enzyme-Linked Immunosorbent Assay; ETS: Environmental Tobacco Smoke; FA: Filtered Air; MCh: Methacholine; PD: Postnatal Days; R<sub>L</sub>: Pulmonary Resistance; SS: Side-Stream Tobacco Smoke; SP: Substance P

### Introduction

ETS exposure in infants and children increases respiratory symptoms such as coughs, wheezing [1] and airway obstruction [2-6]. Epidemiological studies show that children are more susceptible to adverse respiratory effects of passive smoking than adults [7], suggesting that exposure to ETS in early development may be a predisposing factor. Indeed, recent studies in animals showed that exposure to ETS during prenatal and early postnatal life produces the changes to lung function [8], indicating a possible critical period of developmental sensitivity to cigarette smoke exposure during the early postnatal period.

Lung and airways are extensively innervated by sensory nerve system. Stimulation of these sensory endings is known to elicit the release of substance P (SP), which has potent effects on airway smooth muscle tone, vascular permeability and mucus secretion [9-11]. SP plays an important role in antigen or irritant-induced AHR and asthma [12-16]. Our previous study demonstrates that increased SP levels in the airway is involved in cigarette smoke exposure-enhanced airway constriction [8,16]. Nerve growth factor (NGF) is a neurotrophic factor that promotes survival, growth, and differentiation of neurons. Our recent studies have shown that NGF is produced in response to irritant exposures and mediates changes in the phenotype and distribution of SP-containing neurons in the airways [17,18]. Furthermore, it were reported that inflammatory cytokines and chemokines, especially IL-1 $\beta$ , mediated NGF and induced airway hyperresponsiveness [19].

Our recent study showed that early postnatal exposure of mice to side-stream tobacco smoke (SS), a surrogate to environmental tobacco smoke (ETS), leads to increased airway responsiveness and innervation late in life [8]. However, the underlying mechanism initiated in early life that affects airway responses remains. Therefore, the goal of the

experiment is to identify the possible important cytokines and NGF associated with airway hyperresponsiveness, also to determine the changes of airway neurosensory network structures after SS exposure during early prenatal periods.

### Methods

ICR mice (Harlan, Indianapolis, IN) were housed with access to food and water ad libitum in an FDA approved facility. All procedures were performed in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health, and the protocols were approved by the WVU Animal Care and Use Committee #05-0503. The animals were treated humanely and with regard for alleviation of suffering.

### SS exposure

By classical definition, environmental tobacco smoke (ETS) is a diluted mixture of the smoke given off by the burning end of a tobacco product (side stream smoke, ~85%) and the smoke exhaled by smokers (mainstream smoke, ~15%). Base on previous ETS exposure studies [8,20,21], we used side-stream tobacco smoke (SS) as a surrogate to ETS to identify critical developmental periods of susceptibility.

The postnatal days (PD) 2 mice were used to investigate the effects of ETS exposure. Based on our recent study [8], we have used a design where mice were exposed to either SS or filtered air (FA) six hours per day for ten consecutive days beginning on PD2. The SS exposure

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protocol and methods used in this study have been described in our recently publication [8]. Briefly, mice were randomly placed in an exposure chamber (BioClean, DuoFlo, model H 5500, Lab Products Inc) that measured 1.92×1.92×0.97 m (3.58 m<sup>3</sup>). The mice were housed in separate cages located in the exposure chamber. Side-stream smoke from Marlboro filter cigarettes (Phillip Morris, Richmond, VA) was introduced into the exposure chamber at a rate of four cigarettes every 15 minutes for 6 hours per day for 10 days using a smoking machine (RM 1/G, Heinr Borgwald GmbH, Hamburg, Germany). At the end of the 6-hour exposure period, the exhaust fan on the BioClean unit was turned on to rapidly lowered the level of SS in the exposure chamber. The mice were then transported to the animal facilities overnight. The concentrations of carbon monoxide in the exposure chamber were monitored and kept to an average of about 50 parts per million (ppm), relative humidity was about 50%, temperature was about 23°C. Total suspended particulate concentration was about 1.1 mg/m<sup>3</sup>, similar to exposure levels used by others to approximate the cloud of particulates surrounding a person during active smoking [21]. The level of nicotine in blood was also measured in some experiments.

In experiments, mice were randomly divided three groups exposed to either SS or filtered air. First group was tracheostomized for measurement of lung function by total pulmonary resistance and dynamic lung compliance at 1 day, 3 days and 7 days after final SS or FA exposure. Second group was sacrificed and the trachea was removed for immunocytochemistry. Third group was used to Bronchoalveolar Lavage (BAL) fluid by injecting and withdrawing sterile saline via the tracheal cannula. The supernatant of BAL fluid was isolated and stored at -80°C for cytokines and NGF assays and pelleted cells were used to analyze the inflammatory cell.

### Measurements of lung function

Lung function was determined by measuring changes of pulmonary resistance and dynamic compliance after aerosolized methacholine (MCh) (Sigma, St. Louis, MO) challenge using a modification of our previously described [8]. Briefly, mice were anesthetized with pentobarbital (70 mg/kg, intraperitoneally) after SS exposure. The trachea was cannulated just below the larynx via a tracheotomy and a four-way connector was attached to the tracheotomy tube. Two ports were connected to the inspiratory and expiratory tubes of a respirator (Harvard model 683; South Natick, MA). The mice were ventilated at a constant rate of 200 breaths/min and a tidal volume of approximately 0.2 ml. Aerosolized MCh was administered for 30 seconds in increasing concentrations (0, 6.25, 12.5, 25, and 50 mg/ml). For 5 min before and after each MCh challenge, total RL and C<sub>dyn</sub> were analyzed by computer on a breath-by-breath basis.

### Inflammatory cell analysis in Bronchoalveolar Lavage Fluid (BALF)

BALF was obtained by injecting and withdrawing 1ml of sterile saline each time for three times (total 3 ml) via the tracheal cannula at 1 day, or 3 days or 7 days after last SS or FA exposure. The collected BALF (approximately 2.5 ml) was centrifuged at 1,500 rpm for 10 min. The supernatant was frozen at -80°C for cytokines assays, and the pelleted cells were resuspended in a total of 500 µl cold PBS, pooled, plated on glass slides using a cytospin (Shandon Scientific, Ltd., Cheshire, UK) at 400 rpm (18.06 rgf) for 4 min and stained with Wright-Giemsa on a Hema-Tek 2000 automated slide stainer (Bayer, Inc., Tarrytown, NY). A minimum of 200 leukocytes were counted using a light microscope (Olympus AX70) with a 40 times magnification objective.

### Cytokines and NGF Enzyme-Linked Immunosorbent Assay (ELISA)

BALF supernatants were subsequently analyzed by ELISA for IL-1, IL-4, IL-6, IL-10, TNFα and INFγ (BD Biosciences, San Jose, CA), NGF (Promega, Madison, WI) according to the manufacturer's instructions. All samples were run in duplicate or triplicate, and as a negative control, a PBS sample was run with each assay.

### Immunocytochemistry

The procedures for immunocytochemical quantification of airway nerves have been described previously [8,22,23]. Briefly, tracheal segments were removed 1 day, or 3 days or 7 days after the last smoke exposure, and fixed in picric acid-formaldehyde (PAF) fixative for 3 hours, rinsed 3 times with a 0.1 M phosphate-buffered saline containing 0.3% Triton-X-100 (PBS-Tx), frozen in isopentane, cooled with liquid nitrogen, and stored at -80°C. Cryostat sections (12 µm thickness) were collected on gelatin-coated cover slips and dried briefly at room temperature. Then, cryostat sections were covered with rabbit SP antibody (Peninsula Inc, Belmont, CA) diluted 1:100, incubated in a humid chamber at 4°C for 24 hours, rinsed with a 1% bovine serum albumin (BSA)-phosphate saline buffer containing Triton X-100 solution (PBS-Tx plus BSA) three times allowing 5 min for each rinse. The sections were then covered with fluorescein isothiocyanate-labeled goat anti-rabbit antibody (Invitrogen, Carlsbad, CA) diluted 1:100, incubated at 37°C for 30 min, and rinsed. After all immunocytochemical procedures were conducted, the coverslips were mounted with fluoromount and observed under a fluorescence microscope equipped with fluorescein (excitation wavelengths from 455 to 500 nm and emission wavelengths >510 nm) and rhodamine (excitation 540–504 nm, emission >580) filters. Controls consisted of testing the SP specificity of the primary antiserum by absorption with 100 ng/ml of SP. Nonspecific background labeling was determined by omission of primary antiserum.

For measuring nerve fiber density in tracheal smooth muscle, we collected SP nerve fibers in series under the Zeiss LSM 510 confocal microscope. A series of images representing all of the tracheal smooth muscle in a section was collected in digital files and saved to an internal database and measured using Optimas software. We selected regions of smooth muscle using the rhodamine channel to avoid possible bias created by the presence or absence of nerve fibers. The smooth muscle regions were outlined to measure total cross-sectional area of smooth muscle. The microscope was then switched to reveal nerve fibers in the fluorescein channel, and the image was digitally captured. The threshold levels were manually adjusted to subjectively optimize the appearance of fluorescent nerves. The area of nerve fibers was determined by segmentation with the Optimas software. Then nerve fiber area was standardized to the total cross-sectional area of smooth muscle. The final value of nerve fiber density is expressed as % of dividing the SP-nerve fiber area by the total area of smooth muscle. At least 10 measurements were made for each section, and 15 sections were measured in each animal.

### Data analysis

Unless otherwise stated, results are expressed as mean ± SE. The R<sub>i</sub> and C<sub>dyn</sub> elicited by MCh were expressed as a percentage of the baseline. Nerve fiber density was expressed as % area of SP-immunoreactive nerve fibers in the total area of the smooth muscle. Statistical analyses of lung function was performed using one way ANOVA with multiple comparisons, and inflammatory cell and, SP, cytokines and NGF release

in bronchoalveolar lavage fluid were performed using t-test. A *P* value <0.05 was considered significant, and *n* represents the number of animals studied.

## Results

### The changes of lung function after SS exposure during early postnatal period

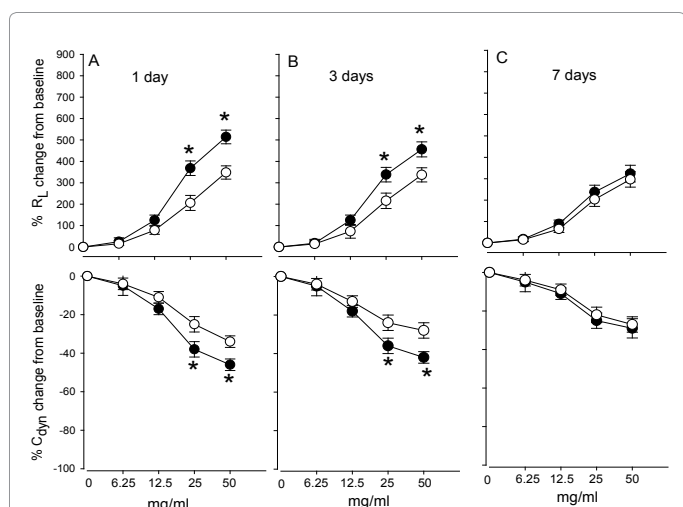
The average nicotine concentration in serum was about 0 ng/mL after FA exposure. Nicotine measured in the SS-exposed animals at the end of the SS exposure was approximately 20 ng/mL [95% confidence interval (CI), 15.3–24.3; *n*=6], which was similar to nicotine levels typically found in human smokers (10–50 ng/mL) [24,25]. After daily exposure to SS or FA for 10 days, there is no significant difference between the weights of the SS exposure and FA mice (10.4 ± 1.1 g vs. 11.8 ± 1.2 g; *p*=0.40; *n*=10, respectively).

The baselines of  $R_L$  and  $C_{dyn}$  in the different groups were not significantly different before MCh challenge (Table 1). However, the MCh dose–response curves were significantly elevated for  $R_L$  and significantly decreased for  $C_{dyn}$  in SS exposed mice (Figure 1) at 1 day and 3 days after SS exposure. For example, a 50 mg/mL MCh dose increased  $R_L$  by 328% (95% CI, 266–390%) at 1 day after FA exposure and by 512% (95% CI, 448–576%; *p*=0.019) at 1 day after SS exposure, and 50 mg/mL MCh increased RL by 317% (95% CI, 253–381%) at 3 days after FA exposure and by 466% (95% CI, 398–534%, *p*=0.035) at 3 days after SS exposure. The significant differences in the  $C_{dyn}$  response to MCh between SS- and FA-exposed mice were found at 1 day and 3 day after exposure (95% CI, –54 to –38% vs. –28 to –36%, *p*=0.038,

	1 day		3 days		7 days	
	$R_L$	$C_{dyn}$	$R_L$	$C_{dyn}$	$R_L$	$C_{dyn}$
SS	1.01 ± 0.18	0.069 ± 0.013	0.99 ± 0.08	0.067 ± 0.014	0.98 ± 0.13	0.066 ± 0.008
FA	0.98 ± 0.14	0.065 ± 0.010	0.97 ± 0.09	0.066 ± 0.012	0.96 ± 0.13	0.065 ± 0.011

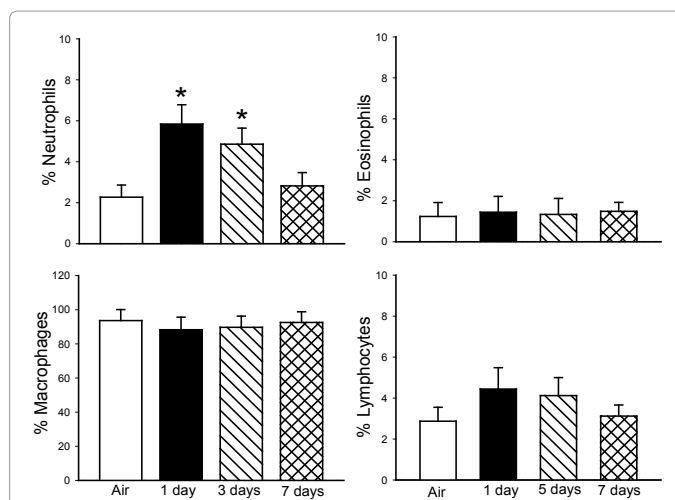
*N* = 6 mice in each group, data are means ± SE

**Table 1:** The baselines of  $R_L$  (cm H<sub>2</sub>O/ml/s) and  $C_{dyn}$  (ml/cm H<sub>2</sub>O) at different times after 10 days SS or FA exposure during early postnatal period



**Figure 1:** MCh dose responses of RL and Cdyn at 1 day (A), 3 days (B) and 7 days (C) after either 10 days SS or FA exposure during early postnatal periods. Data are means ± SE of 6 mice in each group.

\* Significant difference comparing corresponding data between FA and SS animals, *P* ≤ 0.05.



**Figure 2:** The change of leukocyte counts in Bronchoalveolar Lavage Fluid (BALF) at different time after SS exposure. Data are mean ± SE of 6 mice in each group.

\**P* ≤ 0.05, significant difference between FA- and SS-exposed mice.

1 day after exposure and –48 to –36% vs. –34 to –22%, *p*=0.047 3 day after exposure). However, there is no significant difference in  $R_L$  or  $C_{dyn}$  responses to MCh at 7 days after SS exposures. The same MCh dose produced a 324% (95% CI, 256–392%) increase at 7 days after SS exposure and 298% (95% CI, 228–368%, *p*=0.61) increase in FA exposure group (Figure 1C).

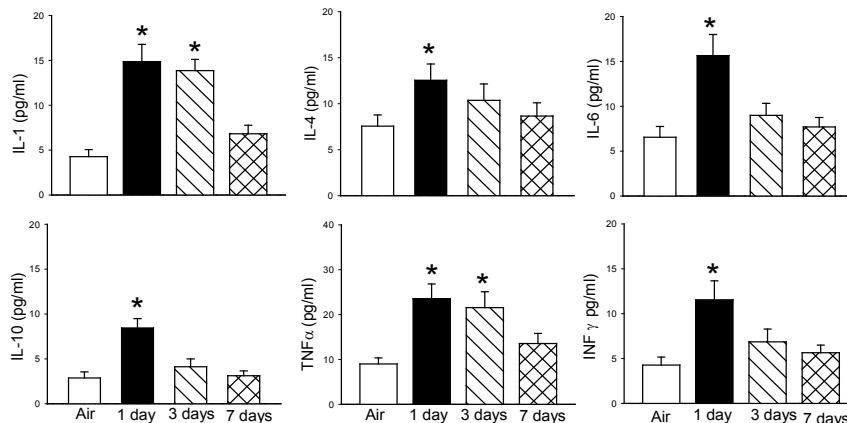
### The changes of airway inflammation and cytokines in BAL fluid after SS exposure

To determine the differences in the BALF inflammatory cell after CS exposure, the leukocytes in the BAL fluid were collected and observed. The total cells were significantly increased at 1 day ( $10.44 \pm 0.67 \times 10^5$ , *p* ≤ 0.001) and 3 days  $8.68 \pm 0.47 \times 10^5$ , *p* ≤ 0.001) after SS exposure compared FA exposure ( $4.36 \pm 0.44 \times 10^5$ ), but the total cells were not significantly different at 7 days ( $5.22 \pm 0.51 \times 10^5$ , *p*=0.24) after SS exposure. A similar trend was also observed for neutrophils. Statistical analyses found that neutrophils were significantly increased in the BAL fluid at 1 day and 3 days after SS exposure (Figure 2), and returned to control level at 7 days after SS exposure. There is no significant difference in eosinophils and lymphocytes (Figure 2).

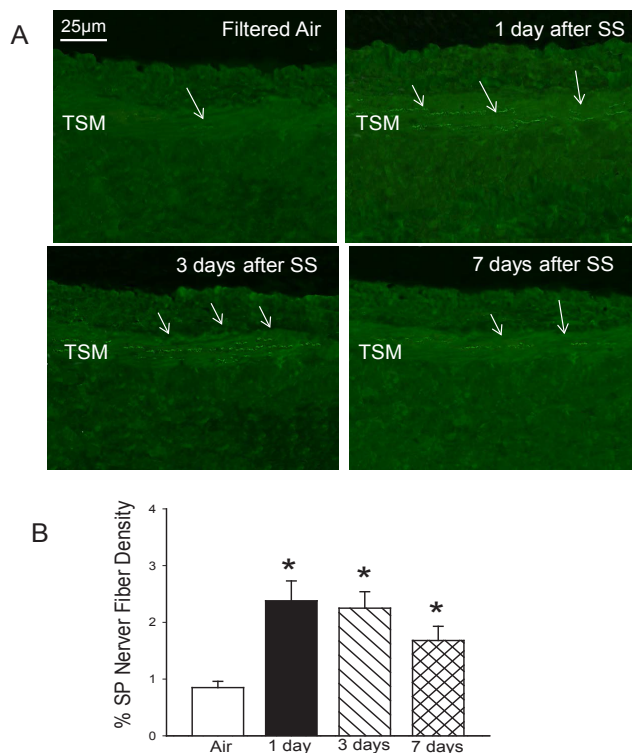
Cytokines including IL-1 $\beta$ , IL-4, IL-6, IL-10, TNF $\alpha$  and INF $\gamma$  in the BALF were further quantified by ELISA at 1 day, 3 days and 7 days after SS exposure. SS exposure significantly increased the concentrations of IL-1, IL-4, IL-6, IL-10, TNF $\alpha$  and INF $\gamma$  at 1 days after SS exposure. The concentrations of IL-1 and TNF $\alpha$  maintained in high levels at 3 days after SS exposure, and returned to control levels at 7 days after SS exposure. However, most of cytokines including IL-4, IL-6, IL-10 and INF $\gamma$  returned to control levels at 3 days after SS exposure (Figure 3).

### The changes of SP-immunoreactive nerve fiber density in tracheal smooth muscle after SS exposure during early postnatal period

To determine the effect of SS exposure on SP innervation, the density of SP nerve fibers in the airway smooth muscle were examined at 1 day, 3 days, and 7 days after SS exposure. The density of SP-immunoreactive nerve fibers in tracheal smooth muscle was significantly increased at 1 day and 3 days after SS exposure compared with FA exposed animals (Figure 4). The density of SP-immunoreactive nerve fibers still



**Figure 3:** The levels of cytokines release in Bronchoalveolar Lavage Fluid (BALF) at different time after SS exposure. Cytokines were measured by ELISA. Data are mean  $\pm$  SE of 6 mice in each group. \* $P \leq 0.05$ , significant difference between FA- and SS-exposed mice.



**Figure 4:** The change of SP nerve fibers in tracheal smooth muscle at different time after SS exposure during early postnatal periods. A: fluorescence photomicrographs of SP nerve fibers in Tracheal Smooth Muscle (TSM) after FA or at 1 days, 3 days and 7 days after SS exposure. B: changes of SP nerve fiber density in tracheal smooth muscle after 10 days FA or at 1 day, 3 days and 7 days after 10 days SS exposure. Arrows: the localization of SP-immunoreactive nerve fibers. Data are mean  $\pm$  SE of 6 mice in each group. \* $P \leq 0.05$ , significant difference between FA- and SS-exposed mice. Magnification:  $\times 285$ .

maintained high level at 7 day after SS exposure (Figure 4B).

### The changes of NGF in BAL fluid after SS exposure during early postnatal period

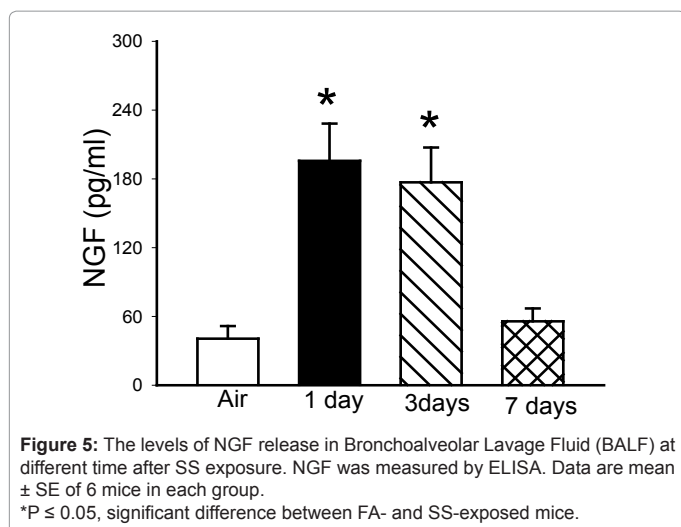
Based on our previous studies [17,18] that NGF production was

stimulated by irritant exposures and mediated the distribution of SP airway innervations, our next experiment focused on the effects of SS on NGF. The concentrations of NGF in BALF were significantly increased at 1 day and 3 days after SS exposure. However, there was no significant difference at 7 days after SS exposure (Figure 5).

### Discussion

Results obtained from the present study show that exposure to SS during early periods of development significantly elevated bronchomotor responses to MCh at 1 day and 3 days after SS exposure. The exaggerated response is accompanied by the enhanced airway inflammatory reaction, as indicated by the marked increases in the level of neutrophils, IL-1 and TNF $\alpha$  et al. in the BALF (Figure 2 and 3). SP and NGF were also significantly elevated after SS exposure during early developmental period. These data confirmed our recent study that the early postnatal period is a period of susceptibility to inhaled SS in mice [8], indicating that exposure to SS during early postnatal period increased SP may associate with the AHR and exaggerated lung inflammation.

ETS exposure in infants and children increases respiratory symptoms and decreases lung function [2,3,21,26-29]. However, most studies only focus on immune mechanisms that contribute to the development of AHR after ETS exposure. Airway sensitivity and airway responsiveness change with age [30-34]. Innervation of the airways and lung are not fully developed in the early postnatal stage of life. The sensitivity of airway nerves in newborns is higher than adult level [35]. Interestingly, the distribution and density of peptidergic nerves in young children is less than adults [36]. Thus, neurotransmitter is age related and opens the possibility that ETS exposure during periods of rapid lung growth and remodeling may alter the normal developmental process. SP acts as a neuromodulator increasing cholinergic sensitivity of airway smooth muscle [37] and the excitability of airway neurons [38]. Recent studies have showed that SP plays an important role in cigarette smoke-induced asthma [12-16]. Several studies have also found that increased SP synthesis is involved in the pathogenesis of human airway diseases [13,39]. These studies show that enhanced SP innervation in human airway sensory nerves may contribute to altered airway function. The current study found that that an initial exposure to SS during early postnatal periods elevated bronchomotor response to MCh, parallel with increased SP level in lung, indicating SP may be



involved in the pathogenesis of SS-induced AHR. Indeed, the studies in our laboratory found that ETS or ozone exposure during early life enhanced SP nerves in airway in rat and mice, accompanied with change of inflammation and pulmonary lung function [8,40]. Cigarette smoke exposure can transiently increase SP in sensory cell bodies projecting to the airways and in axons innervating the airway wall, which modulates cigarette smoke-induced AHR. Although many factors may contribute to early life susceptibility, our studies suggest that detrimental effects of early SS exposures may result in part from altered innervation affecting SP sensory pathways regulating smooth muscle reactivity, bronchial vascular permeability, or mucous secretion.

ETS exposure induces epithelial cell damage and the rapid development of airway inflammation. In the current study, the numbers of neutrophils and cytokines including IL-1 $\beta$ , IL-4, IL-6, IL-10, TNF $\alpha$  and INF $\gamma$  in BAL fluid were increased after SS exposure (Figure 2), indicating that SS exposure-produced acute effects involves enhanced airway inflammation. However, increased neutrophils, cytokines and induced airway hyperresponsiveness returned to control levels at 7 days after SS exposure. These data is parallel with our recent publication [8] which showed that FA re-exposure cannot induce airway hyperresponsiveness in mice exposed SS during early postnatal period, indicating that the late effect of SS exposure in life may involve others. SP is associated with inflammation and interacts with other cells in the lung (e.g., mast cells, leukocytes, epithelial cells) to trigger the release of inflammatory mediators [41]. A recent study found that ETS exposure during early life enhanced SP nerves in bronchial epithelium in infant monkeys, accompanied by increased cytokines in the lung [21], indicating that SP are known to have a wide variety of modulatory effects on inflammatory and immune cell. Thus, it is not surprising in the present study that the initial level of SP and inflammation in BAL fluid parallel significantly increased after SS exposure. It is possible that the SP is increased by early responses subsequent to ETS exposure, promoting immune and smooth muscle responses. Indeed, our study [8] showed that SS re-exposure induced airway hyperresponsiveness and altered airway innervation in adult mice which exposed SS during early postnatal period, suggesting the effect of SS on late in life involved the changes of SP airway neurosensory structures.

Nerve growth factor (NGF) is a neurotrophic factor that promotes and maintains growth of the central and peripheral nervous systems, including those that innervate the airways [42]. NGF in the lung increases during gestation and decreases progressively with postnatal age [43].

Disruption of normal synthesis and release of NGF results in changes in airway innervation which leads to disease-related abnormalities in the respiratory system [17,18,43,44]. Interestingly, recent study found that high level of NGF expression in lung is associated with the development of airway inflammation and AHR [45,46]. We hypothesized that ETS exposure during the early postnatal period alters SP airway innervation, which is possibly mediated by NGF. Current study demonstrated that exposure to ETS during the early postnatal period significantly increased NGF levels in lung, parallel with increased SP and change of lung function after SS exposure; suggesting NGF may regulate SP expression in sensory neurons. There is strong evidence that NGF maintains and even enhances the expression of SP in airway neurons [47]. In mice overexpressing NGF in bronchiolar Clara cells, SP airway innervation is enhanced [48]. Furthermore, our recent study showed that NGF treatment increases SP levels in and around airway neurons and tracheal smooth muscles [22]. These findings, coupled with the current study, support the possibility that NGF serves as a signaling molecule during inflammatory events in the airways by regulating SP production.

In conclusion, the findings of this study show that exposure to SS during early postnatal period significantly induced acute airway hyperresponsiveness and airway inflammation and increased airway innervations. However, increased neutrophils, cytokines and induced airway hyperresponsiveness returned to control levels at 7 days after SS exposure. Combining with our previous publication [8,16], the current study indicates that SS exposure-produced acute effects in lung involve both of airway inflammation and airway innervations, and the late effect of SS exposure during early postnatal mainly involve the change of SP innervation in airway smooth muscle.

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