

Original article

Effects of environmental tobacco smoke on the developing immune system of infant monkeys

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Background: Exposure to environmental tobacco smoke (ETS) is associated with an increased incidence of allergic and infectious diseases among children that is thought to be partly due to the immaturity of the immune system.

Objective: We sought to investigate the effects of ETS exposure on immune development during the first year of life in the nonhuman primate.

Methods: Fifteen neonatal rhesus monkeys studied to 13 months of postnatal age were randomized into 3 groups: (1) exposure to filtered air, (2) continuous ETS exposure beginning at gestation day 50 (perinatal ETS); and (3) exposure to ETS beginning at 6 months of age (6-month ETS). Complete blood counts, lymphocyte subsets, and mRNA levels of 12 cytokines in PBMCs were measured.

Results: Fetal/infant exposure to ETS altered the normal maturation of mRNA levels of IFN- γ , IL-2, and IL-10, as well as the ratio of CD4 to CD8 lymphocytes, compared with filtered-air control levels. Blood lymphocyte subset distribution also significantly differed based on the onset of exposure to ETS. Subacute exposure to ETS for 2 weeks in 6-month-old infants was found to increase levels of peripheral blood neutrophils and IL-6 mRNA.

Conclusions: Short-term exposure to ETS can induce an acute systemic inflammatory response in the neonatal nonhuman primate, and long-term exposure to ETS beginning *in utero* or at 6 months of postnatal age can significantly alter immune effectors.

Clinical implications: Normal immune system development is compromised by *in utero* and postnatal exposure to ETS and might contribute to ETS-related childhood diseases. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Environmental tobacco smoke, immune system maturation, leukocytes, cytokine, gene expression

Abbreviations used

DC:	Dendritic cell
ETS:	Environmental tobacco smoke
FA:	Filtered air
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
NK:	Natural killer
WBC:	White blood cell

Maternal smoking during pregnancy and exposure of infants to environmental tobacco smoke (ETS) is associated with a number of adverse health effects in childhood.¹⁻³ Major sequelae of ETS exposure in infants and children include increased frequency of asthma and lower respiratory tract and middle ear infections.⁴⁻⁶ Whole cigarette smoke, as well as many of its individual constituents, has been shown to have immunomodulatory effects in animals, as well as human fetuses, infants, and adults.⁷⁻¹¹ Previous studies have demonstrated differences in lymphocyte numbers and T-cell cytokine function in newborns compared with adults, which could explain in part the physiologic immaturity and increased susceptibility of the neonatal immune system to the adverse effects of ETS.^{12,13}

Establishing a fully functional immune system requires a series of carefully timed and coordinated developmental events that begin early in embryogenesis and finish during childhood.¹⁴ During infancy and early childhood, the development of the immune repertoire requires exposure to a myriad of antigens, which together stimulate a series of activation and maturation processes.¹⁵ These maturation adjustments continue until an immune balance is achieved that is sufficient to combat the numerous diseases of childhood and adult life.^{15,16} Disruption of immune development by means of perinatal exposure to immunotoxic agents might result in reduced postnatal immune responses or, conversely, exacerbate postnatal aberrant immune responses.¹⁴

Studies of children's exposure to perinatal or postnatal ETS have primarily focused on overall growth and function of the respiratory system.¹⁷⁻¹⁹ Limited information is available about ETS altering the immune system

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during the perinatal or postnatal time period. Our goal is to understand the pathogenesis underlining the disease frequency in children exposed to ETS. Therefore we conducted a controlled longitudinal study in nonhuman primates, a species whose immune system is closely aligned with that of the human, and examined the effect of perinatal and postnatal exposure to ETS on maturational changes of the systemic immune system during the first year of life. Preliminary findings of this study were previously reported in an abstract.²⁰

METHODS

See additional Methods information in the Online Repository at www.jacionline.org.

Animal treatment

Fifteen pregnant rhesus monkeys (*Macaca mulatta*) from the California National Primate Research Center (Davis, Calif) were selected and obtained. Their fetuses/infants were assigned to one of 3 different treatment groups: (1) exposed to filtered air (FA) from gestational day 50 to 13 months of age, (2) exposed to ETS from gestational day 50 (perinatal ETS) to 13 months of age, and (3) exposed to ETS from 6 months of age (6-month ETS) to 13 months of age. Aged and diluted sidestream smoke was used as a surrogate for ETS and produced in a smoking apparatus built in our laboratory, as previously described.²¹ Research cigarettes (2R4F) were obtained from the Tobacco Research Institute at the University of Kentucky. Exposure to ETS occurred for 6 h/d, 5 d/wk at a total suspended particulate concentration of 1 mg/m³.

Blood sampling

Peripheral blood samples were obtained from the infant monkeys at 1, 3, 6, 9, 12, and 13 months of postnatal age. Blood was also drawn 2 weeks after the start of ETS exposure for the 6-month ETS group. All blood samples were processed within 1 hour of collection.

Complete blood counts

EDTA-treated blood was analyzed on an ABX Pentra 60+ electronic cell counter (ABX Diagnostics, Irvine, Calif). The differential count of white blood cells (WBCs) was determined by means of standard counting after Wright-Giemsa staining.

Lymphocyte subsets

Whole blood samples were directly labeled with fluorescein isothiocyanate-conjugated CD3, phycoerythrin-conjugated CD4, peridinin-chlorophyll-protein complex-conjugated CD8, and allophycocyanin-conjugated CD20 mAbs. A FACSCalibur (BD Biosciences, San Jose, Calif) instrument equipped with an automated FACS Loader and CellQuest Software was used for acquisition and analysis of all samples.

Isolation of PBMCs

PBMCs were isolated from heparinized blood by using lymphocyte separation medium and lysed in Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Lysates were frozen at -80°C .

RNA isolation and cDNA preparation

Total PBMC RNA was isolated with Trizol according to the manufacturer's instructions and DNase treated with DNA-free. cDNA was prepared by using random hexamer primers and reverse transcriptase.

Cytokine mRNA analysis by using reverse transcriptase real-time PCR

Twelve cytokines and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were analyzed by using real-time quantitative PCR with an ABI 7700 TaqMan Sequence Detector (Applied Biosystems, Foster City, Calif).²² The primer sequences for each gene are listed in Table E1 (available in the Online Repository at www.jacionline.org). Results are presented as ratios of target mRNA to GAPDH mRNA ($n = 5$).

Statistical analysis

Statistical analysis was performed by using generalized estimating equations and paired t tests in SAS version 8.2 statistical software (SAS Institute, Cary, NC).²³ Analysis of time trends for all immunologic parameters for each group was done by using the slope obtained from generalized estimating equations. Paired t test analysis was used to compare the difference in means between samples before and 2 weeks after initiation of exposure to ETS. Figures were made by using R version 2.0.1 software.²⁴ A P value of less than .05 was considered statistically significant.

RESULTS

Effects of short-term ETS exposure in infant monkeys

To determine whether 2 weeks of ETS exposure induced a systemic inflammatory response, we evaluated complete blood cell counts, lymphocyte subsets, and cytokine mRNA expression in monkeys whose exposure to ETS was initiated at 6 months of age. The absolute numbers and percentages of neutrophils in the blood were significantly higher after 2 weeks of exposure to ETS than before ETS exposure (Fig 1, A and B). The IL-6 mRNA expression level in PBMCs was also increased compared with that before ETS exposure (Fig 1, D). In contrast, the proportion of lymphocytes was significantly lower (Fig 1, C). Other immunologic parameters studied were not affected by short-term exposure to ETS.

Effect of ETS on the cytokine mRNA expression profile in PBMCs during the first year of postnatal life

Cytokine mRNA profiles in PBMCs were characterized at 3, 6, 9, 12, and 13 months of age by means of real-time quantitative PCR to evaluate the effect of perinatal and postnatal ETS exposure on the maturational changes of cytokine expression during the first year of postnatal life. In the FA group mRNA expression of the T_H1 cytokines IFN- γ (Fig 2, A) and IL-2 (Fig 2, D) increased during the first year of life, whereas mRNA expression of the T_H2 cytokines IL-10 (Fig 3, A) and IL-13 (Fig 3, D) decreased. The inflammatory cytokine IL-1 also decreased in expression during the first year of life in the FA group (see Table E2 in the Online Repository at www.jacionline.org).

mRNA expression of IFN- γ during the first year of life was different in the perinatal ETS group, which was exposed to ETS from gestational day 50 to 13 months of age, and the 6-month ETS group, which was exposed to

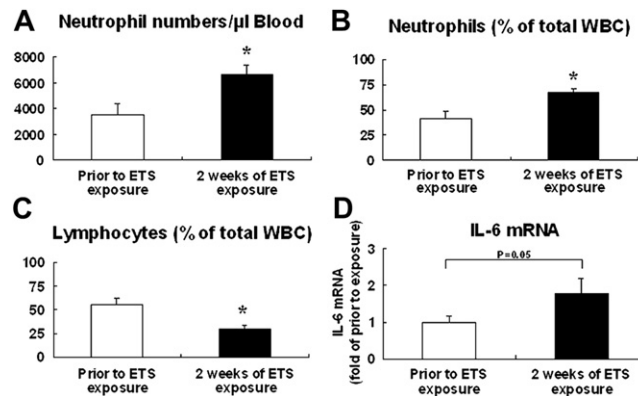


FIG 1. A comparison of systemic markers of inflammation before and after 2 weeks of ETS exposure by 6-month-old monkeys. Absolute number (A) and frequency (B) of neutrophils, frequency of lymphocytes (C), and PBMC IL-6 mRNA levels (D) are shown. Data are presented as means $\pm$ SEs ($n = 5$). * $P < .05$ compared with before ETS exposure.

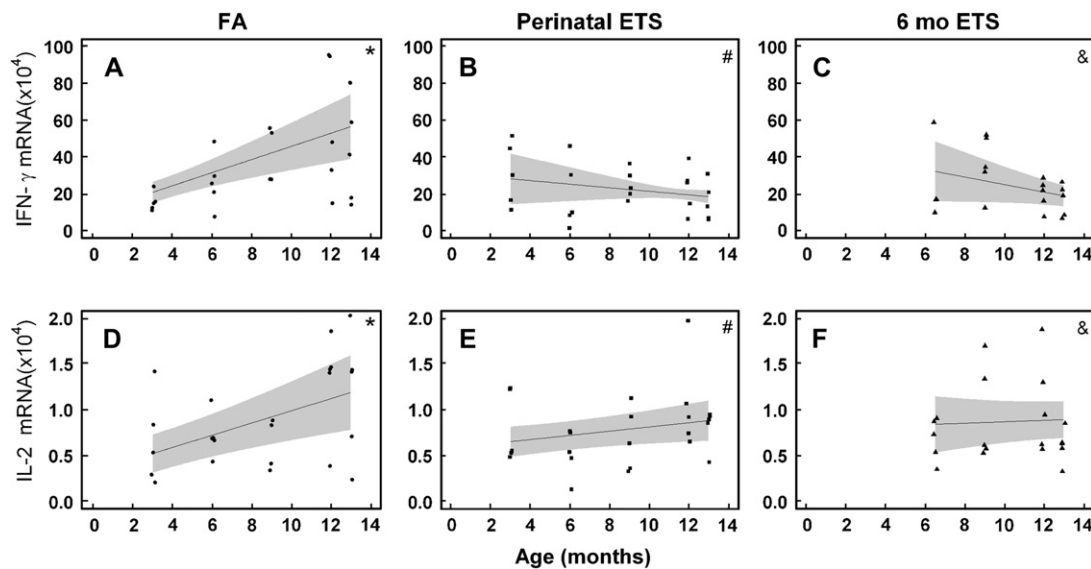


FIG 2. Effect of ETS on IFN- γ and IL-2 mRNA maturation during the first year of postnatal life. Graphs show individual monkeys from the FA (●), perinatal ETS (■), and 6-month ETS (▲) groups; slopes; and 95% CIs (shaded areas). *Age-related changes in FA group, $P < .05$. #Perinatal ETS versus FA, $P < .05$. &6-month ETS vs FA, $P < .05$.

ETS from 6 months of age to 13 months of age, compared with the FA group (Fig 2, A, vs 2, B and C); the 2 ETS groups had progressive decreases in IFN- γ mRNA levels. IL-2 mRNA levels increased with age but to a significantly lesser extent than in the FA group (Fig 2, D, vs 2, E and F). Both ETS treatment groups increased IL-10 mRNA expression during postnatal maturation (Fig 3, A, vs 3, B and C), which was different from the FA group. IL-13 mRNA expression with age was not changed by ETS exposure (Fig 3, D-F). IL-12, IL-4, IL-6, TNF- α , IL-8, monokine induced by IFN- γ , and macrophage inflammatory protein 1 β levels were not affected by ETS (see Table E2 in the Online Repository at www.jacionline.org).

Effect of ETS on postnatal maturational changes in WBC differential

A WBC differential was made by using whole blood samples taken at 1, 3, 6, 9, 12, and 13 months of age to evaluate the effect of ETS on the leukocyte subset distribution during the first year of life. As shown in Table I, for all 3 treatment groups, absolute WBC counts decreased with increasing postnatal maturation. Monkeys starting ETS exposure at 6 months of age had different slopes for absolute numbers and frequencies of neutrophils compared with monkeys in the FA and perinatal ETS groups; 6-month ETS monkeys had decreasing neutrophils, whereas monkeys in the FA and perinatal groups had

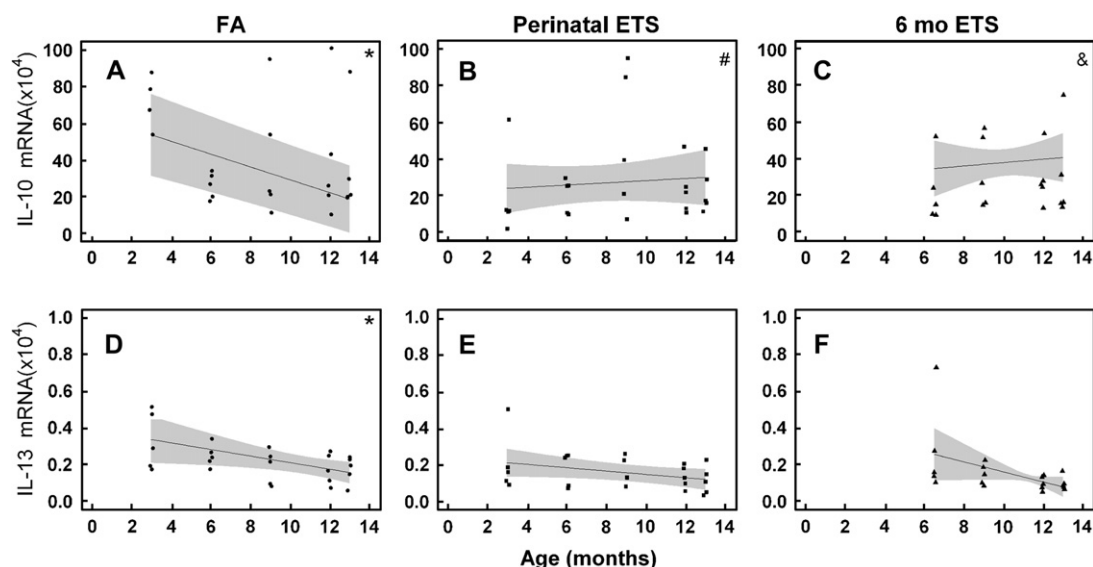


FIG 3. Effect of ETS on IL-10 and IL-13 mRNA maturation in PBMCs. Graphs show individual monkeys from the FA (●), perinatal ETS (■), and 6-month ETS (▲) groups; slopes; and 95% CIs (shaded areas). *Age-related changes in FA group, $P < .05$. #Perinatal ETS versus FA, $P < .05$. &6-month ETS vs FA, $P < .05$.

TABLE I. Estimated slope determined from WBC differential (absolute number and frequency percentage) by using the generalized estimating equation model

WBC differential	FA (control; 1-13 mo)	Perinatal ETS (1-13 mo)	6-mo ETS (6.5-13 mo)
WBC	-0.04 (0.01)*	-0.04 (0.01)*	-0.05 (0.02)*
Neutrophil cells/ μ L	0.02 (0.03)	0.04 (0.01)	-0.09 (0.03)‡§
Percentage of WBC	2.08 (0.50)*	2.82 (0.48)*	-1.49 (0.51)‡§
Lymphocyte cells/ μ L	-0.07 (0.01)*	-0.08 (0.01)*	0.01(0.02)&§
Percentage of WBC	-1.85 (0.47)*	-2.38 (0.54)*	1.68 (0.57)‡§
Monocyte cells/ μ L	-0.06 (0.02)*	-0.14 (0.02)*†	-0.13 (0.06)*
Percentage of WBC	-0.17 (0.07)*	-0.28 (0.07)*	-0.25 (0.11)*
Eosinophil cells/ μ L	-0.11 (0.07)	-0.12 (0.04)	0.04 (0.08)
Percentage of WBC	-0.06 (0.05)	-0.17 (0.05)	0.06 (0.08)

SEs are shown in parentheses.

* $P < .05$ indicates the slope shows an age-related change.

† $P < .05$ indicates the slope is significantly different between the perinatal ETS and FA groups.

‡ $P < .05$ indicates the slope is significantly different between the 6-month ETS and FA groups.

§ $P < .05$ indicates the slope is significantly different between the perinatal ETS and 6-month ETS groups.

increasing neutrophils with age. The opposite was found for lymphocytes. Although both the FA and perinatal groups had a decrease in absolute number and frequency of lymphocytes, the 6-month ETS group had an increase. Peripheral monocytes decreased with age in all 3 groups. However, perinatal ETS monkeys showed a higher rate of decrease in the number of monocytes compared with the FA monkeys. No differences were found for eosinophils.

Effect of ETS on the peripheral blood lymphocyte subsets in the first year of postnatal life

The effect of perinatal or postnatal ETS exposure on the phenotypes of circulating lymphocytes in infant monkeys was made by using whole blood samples taken at 3, 6, 9, and 12 months of age and evaluated by means of flow cytometry. FA monkeys demonstrated an age-related

decrease in T lymphocytes ($CD3^+$; Fig 4, A), B lymphocytes ($CD20^+$; Fig 4, D), and T_H cells ($CD4^+$ T cells; Fig 5, A) during postnatal maturation. Natural killer (NK) cells were evaluated by using the phenotype of $CD3^+CD8^+$, which is generally considered to be appropriate for the identification of NK cells in macaques.²⁵ The absolute number of these cells decreased with age (Fig 4, G) in the FA group. T cytotoxic cells ($CD8^+$ T cells) remained relatively stable (Fig 5, D), and therefore the $CD4/CD8$ ratio decreased during the first year of life in the FA group (Fig 5, G).

The profiles of blood lymphocyte subsets in the perinatal ETS monkeys were similar to those in the FA group (Figs 4 and 5), except for the $CD4/CD8$ ratio (Fig 5, H). Compared with the FA and perinatal ETS groups, monkeys that started ETS exposure at 6 months of age had a significantly different peripheral blood lymphocyte subset

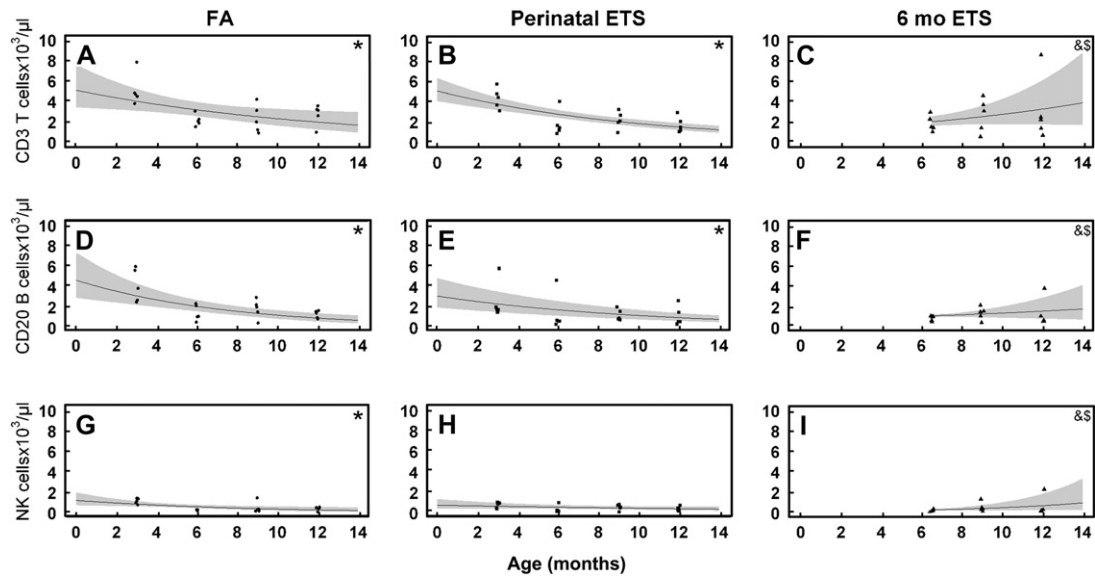


FIG 4. Effect of ETS on lymphocyte maturation during the first year of postnatal life. Graphs show individual monkeys from the FA (●), perinatal ETS (■), and 6-month ETS (▲) groups; slopes; and 95% CIs (shaded areas). *Age-related changes, $P < .05$. &6-month ETS versus FA, $P < .05$. \$Perinatal ETS versus 6-month ETS, $P < .05$.

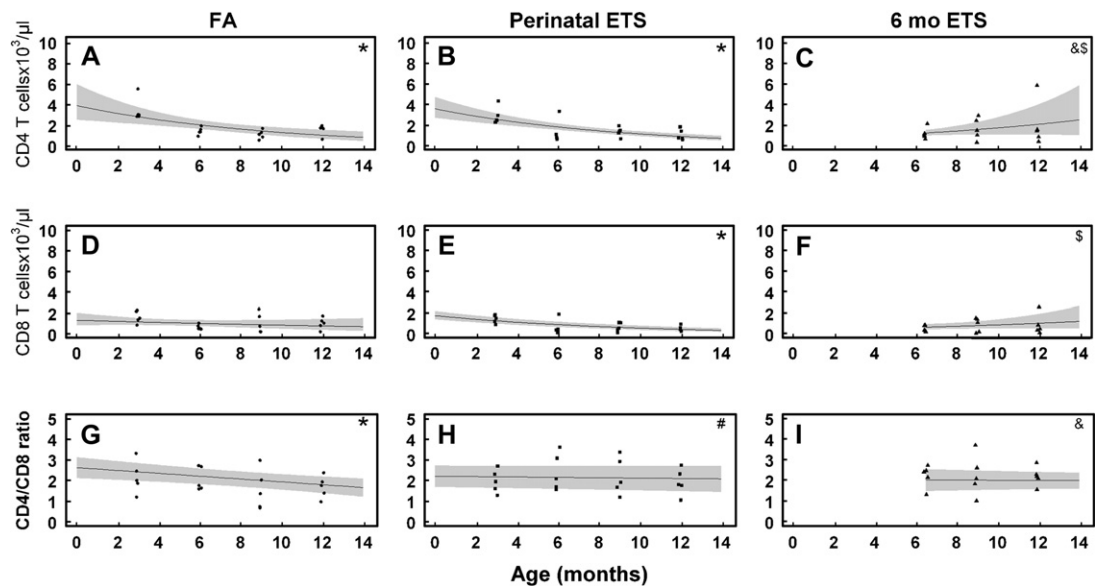


FIG 5. Effect of ETS on T-lymphocyte maturation during the first year of postnatal life. Graphs show individual monkeys from the FA (●), perinatal ETS (■), and 6-month ETS (▲) groups; slopes; and 95% CIs (shaded areas). *Age-related changes, $P < .05$. &6-month ETS versus FA, $P < .05$. \$Perinatal ETS versus 6-month ETS, $P < .05$.

for $CD3^+$ T cells, $CD20^+$ B cells, NK cells, and $CD4^+$ T cells (Fig 4, C, F, and I, and Fig 5, C, respectively).

DISCUSSION

Here we show that perinatal and postnatal exposure to ETS alters maturational changes of specific immunologic parameters within the peripheral blood during the first year

of life. The level of ETS used in this study was based on measured concentrations of cigarette smoke surrounding a person (eg, a caregiver) engaged in the act of smoking.²⁶ To our knowledge, this is the first report to reveal short and long-term effects of ETS on the developing immune system in a primate species.

The significant increase in the peripheral neutrophils and IL-6 mRNA observed in PBMCs after the 6-month group was exposed to ETS for 2 weeks strongly suggests

that short-term ETS exposure in infant monkeys induces a systemic inflammatory response. Adult cigarette smokers have increases in peripheral leukocyte counts and neutrophilia in lung tissue.^{27,28} Free radicals found in cigarette smoke could be responsible for increasing IL-6 mRNA in PBMCs. Reactive oxygen species can activate nuclear factor κ B and activator protein 1 and thus activate the genes of proinflammatory mediators, such as IL-6.²⁹ The effect of 2 weeks of ETS exposure on neutrophils and lymphocytes did not persist further in monkeys starting ETS exposure at 6 months of age. Rather, there was a progressive decrease in neutrophils and an increase in lymphocytes from 6.5 to 13 months of age. The mechanisms for these changes are unknown; however, systemic inflammation might be a factor involved in initiating adverse health effects from subacute ETS exposure during childhood.

Normal maturation of peripheral leukocytes has been reported for both human subjects and rhesus monkeys.^{25,30} Our results are consistent with previous findings that WBC counts and the number of peripheral blood lymphocytes progressively decrease during infancy in monkeys. Surprisingly, we did not see significantly different changes in WBC differential and lymphocyte subsets with ETS exposure, except for a faster decrease of monocytes in infants exposed to ETS *in utero*. Increased migration and differentiation of monocytes into the pulmonary system might be responsible. Chronic cigarette smoking has been associated with increased numbers of macrophages in bronchial lavage fluid.³¹ The reasons for the observed difference in developmental patterns of leukocyte populations and specific lymphocyte subsets between the perinatal ETS and 6-month ETS groups are not clear. The maturational status of the immune system of these monkeys when ETS exposure is initiated might play a role.

Cytokines are important in orchestrating inflammatory and immunologic effector mechanisms. In the present study we analyzed the *in vivo* expression of typical type 1 cytokines (IFN- γ , IL-2, and IL-12) and type 2 cytokines (IL-4, IL-10, and IL-13), which are key cytokines determining the T_H1 and T_H2 immune response and favoring the cellular or humoral immune response, respectively. We also investigated the cytokines IL-1, IL-6, and TNF- α and the chemokines IL-8, monokine induced by IFN- γ , and macrophage inflammatory protein 1 β , all of which play a role in innate immunity and the adaptive immune response.²² Our results confirm previous reports that T cells capable of IFN- γ production increase progressively with age^{15,32} and also demonstrate age-related increases in IL-2 mRNA expression during infancy. Furthermore, mRNA expression levels of the T_H2 cytokines IL-10 and IL-13 progressively decreased during the first year of postnatal life, which is consistent with previous reports³³⁻³⁵ describing an age-dependent decrease in IL-10 and IL-13 release in response to inhalant allergens in cord blood mononuclear cells and PBMCs of healthy children. These findings suggest a progressive maturation of the T_H1 pattern with increasing age during postnatal life in FA-exposed monkeys. In contrast, we observed an

inhibition in IFN- γ and IL-2 and an increase in IL-10 mRNA levels in the perinatal ETS group and the infants beginning exposure to ETS at 6 months of age. Impaired T_H1 cytokine maturation coupled with the increase in T_H2 cytokine levels observed in our study suggest that ETS alters the balance of T_H1/T_H2 cytokine secretion and skews the immune response toward the allergy-associated T_H2 cytokine phenotype in infancy.

Generalized functional immaturity of the immune system during infancy is particularly manifested in the T_H1 arm of the adaptive immune response.^{36,37} Our findings suggest that ETS exposure delays or reduces the efficiency of T_H1 maturation postnatally. A lack of development of T_H1 responses, a blunting of T_H1 responses, or both might leave the individual with a predominant T_H2 response and predisposes them to atopy.³⁸ Atopy in 12-year-old children was found to be most prevalent among those who previously did not have T_H1-dependent delayed tuberculin hypersensitivity in response to BCG vaccination during infancy.³⁹ The T-cell selection process is dependent on the functions of relevant antigen-presenting cells. Principal antigen-presenting cell populations involved are intraepithelial dendritic cells (DCs), which occur as a dense network throughout the airway mucosa.⁴⁰ DC populations from central lymphoid organs in newborn animals are deficient in the capacity to prime naive T cells for T_H1-dependent memory generation, resulting in a generalized skewing of immune responses during infancy toward the T_H2 cytokine profile.⁴¹ Previous studies have reported that the antibody response to a variety of antigens, such as influenza virus infection and vaccination, is decreased in cigarette smokers.⁴² Seymour et al⁴³ have demonstrated that tobacco smoke might enhance the development of the T_H2 response in a murine model of allergy. The ability of cigarette smoke to impair antigen-presenting cell function by inhibiting the ability of DCs to stimulate appropriate T-cell responses has also been reported recently.⁴⁴

All findings here were obtained within blood and might not parallel immunopathologic changes in the respiratory tract. However, findings in a neonatal mouse model provide evidence that sidestream cigarette smoke exposure leads to a significant decrease in IL-12 and IFN- γ levels in the lungs.⁴⁵ Our preliminary studies also suggest that exposure to ETS alters the T_H1/T_H2 balance toward a T_H2 phenotype in lung tissues, which is consistent with the changes in the blood observed in this study (data unpublished). The ability of ETS alone to induce an asthmatic condition is still not clearly established; whether there is a causal relationship or an adjuvant effect remains to be determined. However, we have previously reported that ETS exposure during the perinatal period suppresses nuclear factor κ B signaling to increase apoptosis, which might affect the overall growth and function of the respiratory system.⁴⁶ Changes in cytokine patterns and peripheral leukocyte numbers are highly suggestive of altered immune development caused by exposure to ETS, but further studies are required for confirmation, especially in regard to whether such changes at 1 year of postnatal life persist and result in asthma or long-term adult-onset

disease, such as chronic obstructive pulmonary disease, emphysema, or cancer.

In summary, our findings suggest that exposure to low levels of ETS during critical windows of immune development alter cytokine response and immune effectors, which might compromise normal immune system development, with potential implications for future onset of diseases. These findings further indicate that rhesus monkeys can provide a valuable nonhuman primate model to examine environmental influences on the functional immune response in the neonate.

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