

# Effects of environmental tobacco smoke exposure on pulmonary immune response in infant monkeys

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**Background:** Exposure to environmental tobacco smoke (ETS) in early life has adverse effects on lung development and increases asthma incidence and susceptibility to infection. We have previously reported that perinatal and postnatal exposure to ETS in infant monkeys leads to an impaired T<sub>H</sub>1 immune response in peripheral blood.

**Objective:** Determine whether ETS exposure during the perinatal period alters pulmonary immune maturation in the neonatal lung.

**Methods:** Nonhuman primates were exposed to ETS from gestation day 50 to 13 months postnatal age (perinatal ETS) or from 6 to 13 months (postnatal ETS). Control animals were only exposed to filtered air. T<sub>H</sub>1 and T<sub>H</sub>2-related cytokines, chemokines, and their corresponding receptors as well as transcription factors were analyzed in lung tissues at 13 months.

**Results:** Animals exposed to ETS beginning *in utero* exhibited more profound alterations in T<sub>H</sub>1 factors compared with animals exposed to ETS beginning at 6 months postnatal age. In perinatal ETS-exposed monkeys, mRNA for IFN- $\gamma$ , IL-2, IFN- $\gamma$ -inducible protein 10, monokine induced by IFN- $\gamma$ , IFN- $\gamma$ -inducible T-cell chemoattractant, CXC chemokine receptor 3, IL-12 bioactive p70 subunit, and T-bet were significantly downregulated, whereas in postnatal ETS-exposed monkeys, only IFN- $\gamma$ , CXC chemokine receptor 3, and IL-12p70 were significantly downregulated. ETS effects on T<sub>H</sub>2 factors were less apparent and more variable: mRNA for thymus and activation-regulated chemokine was increased, and IL-10 protein was reduced.

**Conclusions:** Environmental tobacco smoke exposure during early life enhances a local T<sub>H</sub>2 immunity by impairing normal pulmonary T<sub>H</sub>1 immune maturation. This effect was greater in animals beginning ETS exposure *in utero*. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

**Key words:** Environmental tobacco smoke, lung development, T<sub>H</sub>1 immune response

## Abbreviations used

|           |                                                 |
|-----------|-------------------------------------------------|
| CXCR3:    | CXC chemokine receptor 3                        |
| ETS:      | Environmental tobacco smoke                     |
| FA:       | Filtered air                                    |
| GAPDH:    | Glyceraldehyde-3-phosphate dehydrogenase        |
| IL-12p70: | IL-12 bioactive p70 subunit                     |
| IP-10:    | IFN- $\gamma$ -inducible protein 10             |
| ITAC:     | IFN- $\gamma$ -inducible T-cell chemoattractant |
| MIG:      | Monokine induced by IFN- $\gamma$               |
| TARC:     | Thymus and activation-regulated chemokine       |

The pulmonary immune system is an interconnecting network of immune structures that provides a first line of defense to the airway mucosal surface. Maturation of the mucosal immune system and the establishment of protective immunity vary between individuals but are usually fully developed in the first year of life, irrespective of gestational age at birth.<sup>1</sup> Any environmental insult, such as environmental tobacco smoke (ETS), during this period has the potential to affect significantly the overall growth and function of the respiratory system.<sup>2</sup> There is mounting evidence that when infants and children are exposed to ETS, they increase their risk of developing asthma and lower respiratory tract infections.<sup>3-5</sup> The increased susceptibility has been related to the immaturity of the neonatal immune system during ETS exposure, especially the pulmonary compartment of the immune system.

Optimal clearance of antigens encountered by the airway requires the selective activation of particular cellular and/or humoral immune responses. The balance of T<sub>H</sub>1 or T<sub>H</sub>2 cytokines determines the type of effector response. Recent studies suggest a number of other molecules, including chemokines, chemokine receptors, and transcription factors, can also influence the immune response by activation and differentiation of different cell populations involved in allergic diseases, such as T<sub>H</sub>1 and T<sub>H</sub>2 cells.<sup>6-10</sup> The transcription factors T-bet and GATA-3 are important reciprocal determinants of T<sub>H</sub>1 and T<sub>H</sub>2 cell differentiation. Recent evidence suggests that these factors may affect airway immunopathology in asthma.<sup>11</sup> Generalized functional immaturity of the immune system during infancy is particularly manifested in the T<sub>H</sub>1 arm of the adaptive immune response.<sup>12,13</sup> A lack of development and/or a blunting of T<sub>H</sub>1 responses may leave individuals with a predominant T<sub>H</sub>2 response and predispose them to atopy.<sup>14</sup> Decreased IL-12 bioactive p70 subunit (IL-12p70) production,<sup>15,16</sup> elevated type 2 chemokine thymus and activation-regulated chemokine (TARC) expression,<sup>17</sup> and enhanced T<sub>H</sub>2 immune response<sup>18</sup> by cigarette smoke or its components have been reported previously, which suggests ETS exposure may alter the normal T<sub>H</sub>1/T<sub>H</sub>2 balance, leading to the development of allergic or infectious diseases. Currently, very little data are available on the effects of ETS exposure during the perinatal or postnatal period on the

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expression of  $T_H1/T_H2$  related molecules (eg, cytokines), particularly with respect to *in situ* production in the lungs.

We have previously described how exposure to ETS during early life by neonatal monkeys alters the systemic immune system by delaying the efficiency of  $T_H1$  maturation in peripheral blood.<sup>19</sup> The current study extends this research to investigate the cytokine profile of the pulmonary immune system. We examined lung tissue from the same animals used in the systemic immune study<sup>19</sup> to examine the effect of ETS exposure on the expression of molecules affiliated with  $T_H1$  or  $T_H2$  responses. Our data demonstrate that fetal/postnatal exposure to ETS impairs normal pulmonary type 1 immune maturation.

## METHODS

For additional information, see this article's [Methods](#) section in the Online Repository at [www.jacionline.org](http://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 3 different treatment groups: (1) exposed to filtered air (FA;  $n = 5$ ) from gestational day 50 to 13 months of age, (2) exposed to ETS from gestation day 50 (perinatal ETS;  $n = 5$ ) to 13 months of age, and (3) exposed to ETS from 6 months of age (postnatal ETS;  $n = 5$ ) 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.<sup>20</sup> 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  $1 \text{ mg/m}^3$  (see this article's [Table E1](#) in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). The measurements of nicotine and cotinine levels in plasma confirmed that the filtered air group had a very low level of nicotine ( $<1 \text{ ng/mL}$ ) and undetectable cotinine, whereas the perinatal ETS and postnatal ETS groups showed comparable plasma nicotine (6.6 ng/mL) and cotinine levels (70 ng/mL).

### Necropsy and tissue sampling

An overdose of pentobarbital (60 mg/kg) was administered by intravenous injection to each infant the day after the termination of ETS exposure. The superior and inferior lobes of the right lung were removed and frozen. The left lung and the right middle lobe were fixed and stored in fixative. Fixed tissue samples were embedded in paraffin, sectioned, and stained to examine the airways and parenchyma.

### Quantitative real-time PCR

Total RNA was isolated from lung tissue, and cDNA synthesis was performed. Primers of IFN- $\gamma$ , IL-2, IL-4, IL-13, chemokine CXCR3 (CXCR3), CCR5, IFN- $\gamma$ -inducible protein 10 (IP-10), monokine induced by IFN- $\gamma$  (MIG), IFN- $\gamma$ -inducible T-cell chemoattractant (ITAC), CCR3, CCR4, eotaxin, TARC, T-bet, GATA-3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are described in this article's [Table E2](#) in the Online Repository at [www.jacionline.org](http://www.jacionline.org). Quantitative detection of GAPDH and target genes was performed with a LightCycler Instrument (Roche Diagnostics, Indianapolis, Ind).<sup>21</sup> Results are presented as ratios of target mRNA to GAPDH mRNA.

### Immunohistochemical staining of CXCR3 in the airways

Briefly, paraffin-embedded sections were taken through xylene, ethanol, and AR-10 antigen retrieval solution. Sections were incubated in anti-CXCR3 antibody and subsequently incubated in goat antimouse IgG, horseradish peroxidase, and diaminobenzidine substrate. Sections were counterstained with hematoxylin. Semiquantitative assessment of CXCR3-positive cells was determined for both the subepithelium and the parenchyma.

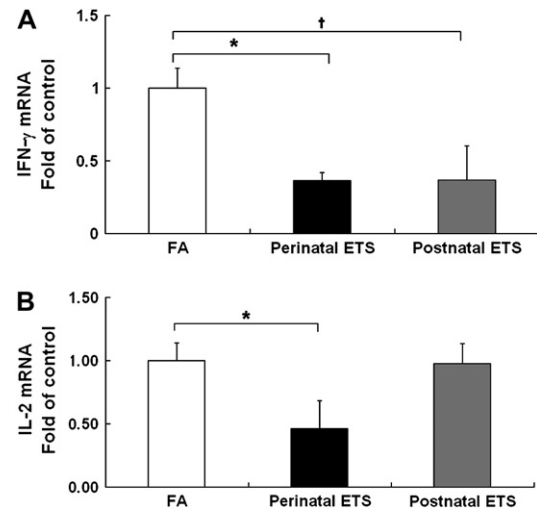


**FIG 1.** Relative levels of expression of mRNA for IFN- $\gamma$  (A) and IL-2 (B) in lung tissue from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. The data are expressed as the means  $\pm$  SEMs ( $n = 5$ ). \*Perinatal ETS compared with FA,  $P < .05$ . †Postnatal ETS compared with FA,  $P < .05$ .

### IL-10 and IL-12p70 detection by ELISA

The concentrations of monkey IL-10 and IL-12p70 in the lung tissue homogenate were determined by ELISA kits according to the manufacturer's protocols.

### Statistical analysis

All data were presented as means  $\pm$  SEMs. Comparisons among ETS-exposed and FA-exposed groups were made by 1-way ANOVA followed by *post hoc* Fisher test. A value of  $P < .05$  was considered significantly different.

## RESULTS

### ETS effects on cytokine and chemokine mRNA expression in the infant lung

The expression of  $T_H1$  cytokines IFN- $\gamma$  and IL-2 and  $T_H2$  cytokines IL-4 and IL-13 was examined by quantitative real-time PCR and compared among groups. In comparison with the FA control, both perinatal and postnatal exposure to ETS significantly decreased IFN- $\gamma$  mRNA expression in lung tissue (Fig 1, A). IL-2 mRNA expression was significantly suppressed in the perinatal ETS group (Fig 1, B).  $T_H2$  cytokines were not affected by ETS exposure (see this article's [Fig E1](#) in the Online Repository at [www.jacionline.org](http://www.jacionline.org)).

We also examined the expression of purported  $T_H1$ -affiliated and  $T_H2$ -affiliated chemokines in lung tissue. The expressions of  $T_H1$  affiliated chemokines IP-10, MIG, and ITAC were significantly downregulated in the perinatal ETS group compared with the FA control (Fig 2, A-C), whereas the expression of  $T_H2$ -affiliated TARC was significantly upregulated in the perinatal ETS group (Fig 2, D). The expressions of IP-10, MIG, ITAC, and TARC in the postnatal ETS group were greater than the FA control but were not statistically significant.  $T_H2$  chemokine eotaxin was not affected by ETS exposure (see this article's [Fig E2](#) in the Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### ETS effects on the mRNA expression of chemokine receptors in the lung

Different patterns of chemokine receptor expression have been associated with  $T_H1$ -polarized and  $T_H2$ -polarized cells.  $T_H1$  cells

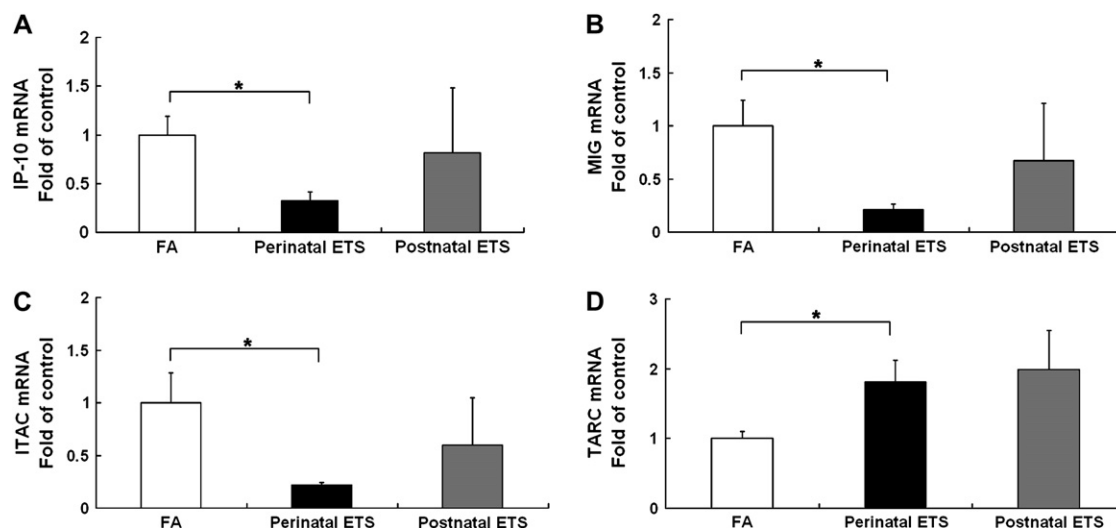


**FIG 2.** Relative levels of mRNA expression for IP-10 (A), MIG (B), ITAC (C), and TARC (D) in lung tissue from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. The data are expressed as the means  $\pm$  SEMs ( $n = 5$ ). \*Perinatal ETS vs FA,  $P < .05$ .

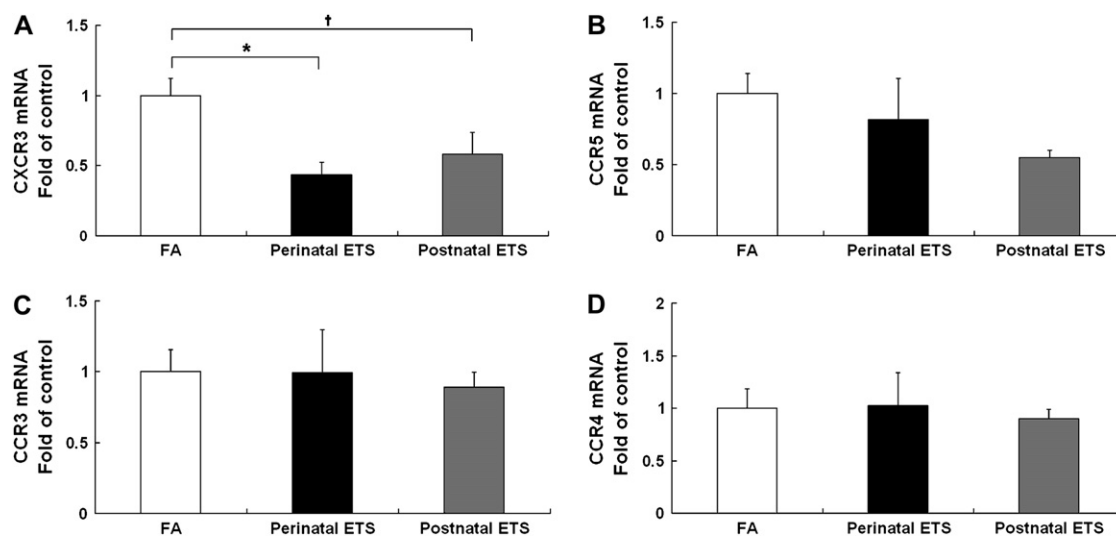


**FIG 3.** The effect of ETS exposure on the mRNA expression of CXCR3 (A), CCR5 (B), CCR3 (C), and CCR4 (D) in the lung from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. The data are expressed as the means  $\pm$  SEMs ( $n = 5$ ). \*Perinatal ETS vs FA,  $P < .05$ . †Postnatal ETS vs FA,  $P < .05$ .

preferentially express CCR5 and CXCR3, whereas CCR3 and CCR4 are expressed on  $T_H2$  cells.<sup>22</sup> Here we examined the expression of CXCR3, CCR5, CCR3, and CCR4 in lung tissue. As seen in Fig 3, A, CXCR3 expression was downregulated in both perinatal and postnatal ETS groups relative to FA control. mRNA expression for CCR5, CCR3, and CCR4 was not affected by ETS (Fig 3, B-D).

### ETS effects on the recruitment of CXCR3-bearing immune cells in the lung

To examine whether the decreased  $T_H1$  cytokine and chemokine expression was associated with effects of ETS on the recruitment of type 1 immune cells into the lung, we immunohistochemically stained for CXCR3 on lung sections. The majority of CXCR3-positive cells were located in the intraepithelial and

subepithelial areas (Fig 4, A-C). Compared with the FA control, there was a significant decrease in the number of CXCR3<sup>+</sup> cells in the subepithelial areas in both perinatal and postnatal ETS groups (Fig 4, A, vs Fig 4, B and C, and Fig 5), which was consistent with the mRNA expression in the lung tissue. However, the number of CXCR3<sup>+</sup> cells in the alveolar tissues was not significantly different among groups (data not shown).

### ETS effects on the regulatory cytokines IL-12p70 and IL-10

IL-10 and IL-12 are considered to affect  $T_H1/T_H2$  balance by regulating IFN- $\gamma$  production. We measured the synthesis of bioactive IL-12p70 and IL-10 in the lung tissue. The concentration of IL-12p70 was significantly decreased in both perinatal and postnatal ETS groups (Fig 6, A). The concentration of IL-10

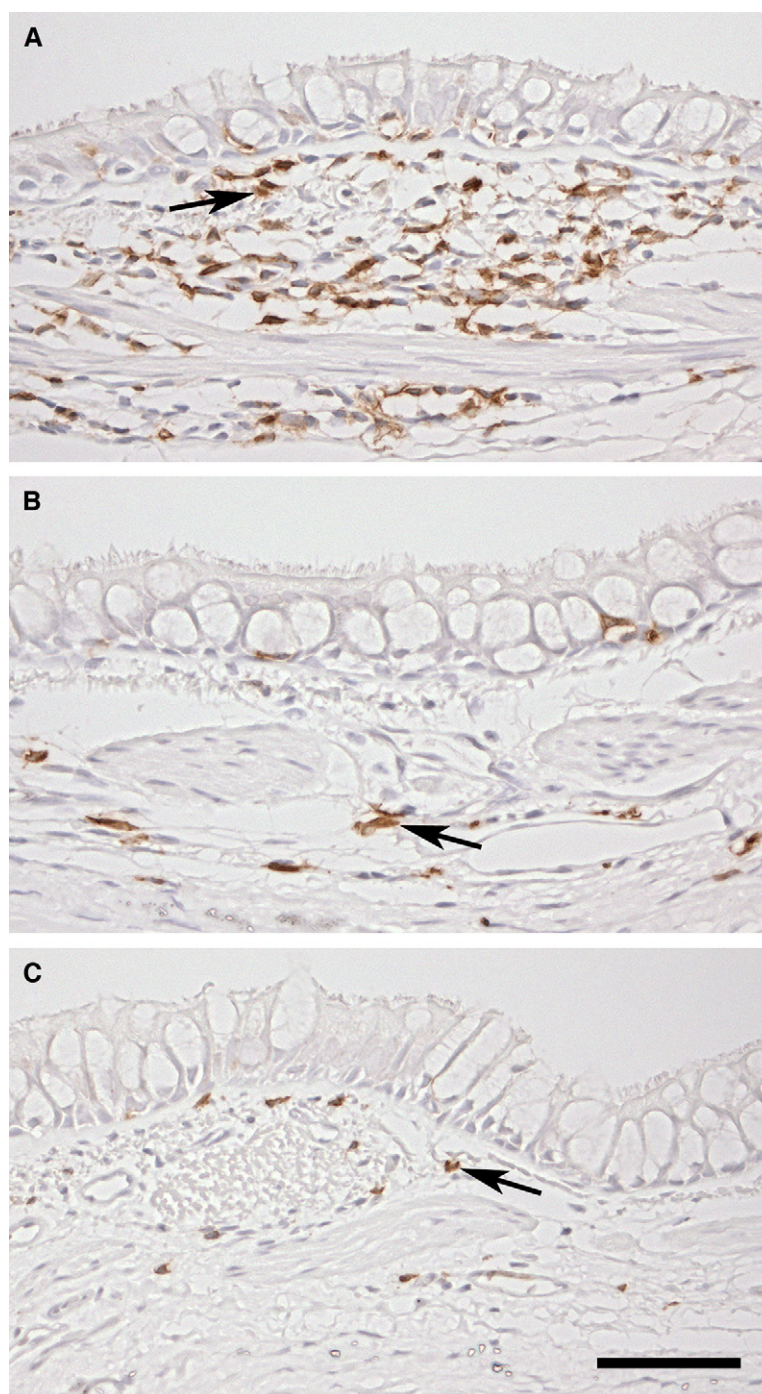


**FIG 4.** Localization of CXCR3-positive cells in the airway. **A**, The subepithelium of a representative FA-exposed control lung has immune cells immunostained positive for CXCR3 (arrow). **B**, Lung tissue from a perinatal ETS-exposed monkey. **C**, Lung tissue from a postnatal ETS-exposed monkey. Magnification bar: 50  $\mu$ m.

was also significantly decreased in both ETS treatment groups compared with the FA control (Fig 6, B).

#### ETS effects on transcription factor expression

To investigate whether the changes in cytokine and chemokine expression described were associated with modulating effects of ETS on the expression of transcription factors, we examined T-bet and GATA-3 expression in the lung tissue. Fig 7, A, demonstrates that t-bet expression was significantly downregulated only in the

perinatal ETS group compared with the FA group. There was a trend of decrease in T-bet mRNA expression in the postnatal ETS group, although not statistically significant. GATA-3 expression was not affected by ETS exposure (Fig 7, B).

#### DISCUSSION

In the current study, the cytokine and chemokine profiles as well as transcription factors were compared between infant

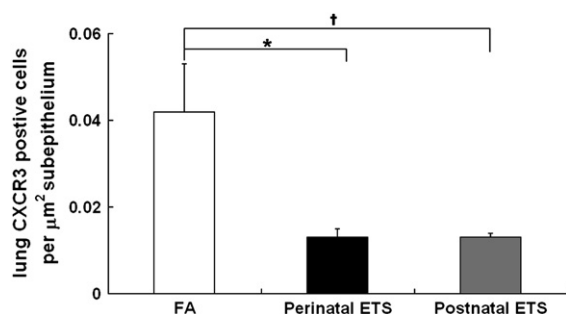


**FIG 5.** The effect of ETS exposure on the number of CXCR3-positive cells in the subepithelium. The number of positive cells per square micrometer subepithelium in lung tissue from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. Data are expressed as the means  $\pm$  SEMs (n = 5). \*Perinatal ETS vs FA,  $P < .05$ . †Postnatal ETS vs FA,  $P < .05$ .

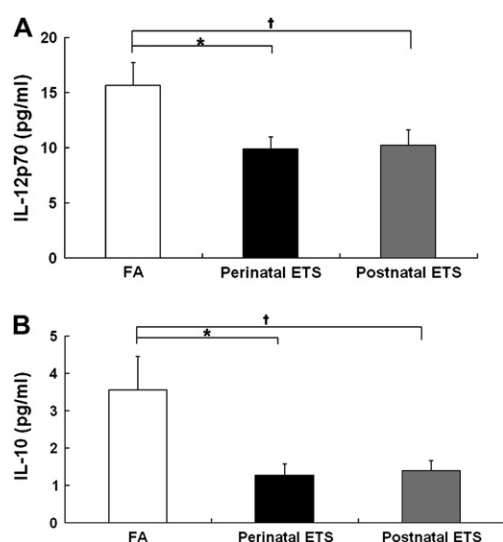


**FIG 6.** The effect of ETS exposure on the regulatory cytokines IL-12p70 (A) and IL-10 (B) in the lungs from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. The data are expressed as the means  $\pm$  SEMs (n = 5). \*Perinatal ETS vs FA,  $P < .05$ . †Postnatal ETS vs FA,  $P < .05$ .

monkeys with perinatal ETS exposure, monkeys with only postnatal ETS exposure, and FA controls. We found that animals exposed to ETS beginning *in utero* exhibited more profound changes in the parameters examined compared with the animals exposed to ETS beginning at 6 months postnatal age. In perinatal ETS-exposed monkeys, a total of 11 altered parameters (IFN- $\gamma$ , IL-2, IP-10, MIG, ITAC, TARC, CXCR3 mRNA, CXCR3-positive cells, IL-12p70, IL-10, and T-bet) were found—more than twice that found in postnatal ETS-exposed monkeys (IFN- $\gamma$ , CXCR3, CXCR3-positive cells, IL-12p70, and IL-10). These differences may be the result of variations in animal susceptibility at different ages and/or stages of development during exposure, the route of exposure (direct vs indirect inhalation during postnatal vs gestational), and the duration of exposure.

We have previously shown that perinatal and postnatal exposure to ETS by infant monkeys leads to an impaired  $T_H1$  immune response in the peripheral blood.<sup>19</sup> However, at that time, we did not fully investigate the cytokine profile of the pulmonary immune system. In this study, we examine the effect of perinatal and postnatal ETS exposure on mRNA or protein expression of various

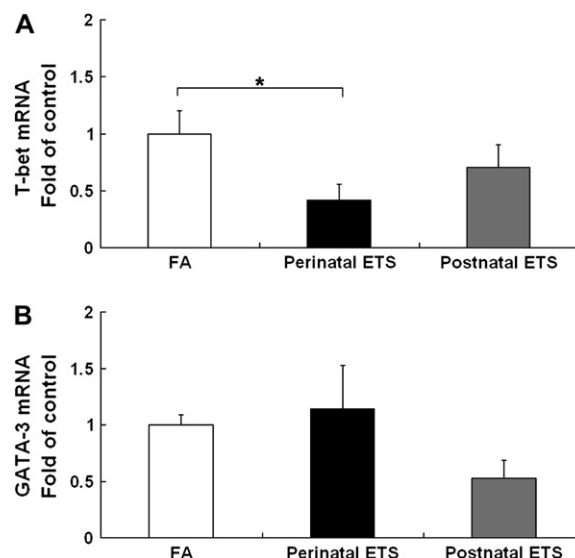


**FIG 7.** Relative levels of mRNA expression for transcription factor T-bet (A) and GATA-3 (B) in lung tissues from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. The data are expressed as the means  $\pm$  SEMs (n = 5). \*Perinatal ETS vs FA,  $P < .05$ .

$T_H1$  and  $T_H2$ -related cytokines, chemokines, and transcription factors in the lung tissue. We also examine the location of cells expressing a  $T_H1$ -associated receptor. In general, we found that the  $T_H1$ -associated mRNA and protein were reduced by perinatal ETS exposure and sometimes also by postnatal ETS exposure. In contrast,  $T_H2$ -associated mRNA and protein were generally not altered by perinatal or postnatal ETS exposure. Thus, the balance of  $T_H1$  versus  $T_H2$  is tipped in favor of  $T_H2$ ; this result favors the potential for atopic diseases, such as asthma, to develop.

Alterations in the intensity or the type of immune responses can have a profound effect on respiratory disease.  $T_H1$  cells promote cell-mediated and inflammatory responses through their production of cytokines, such as IL-2 and IFN- $\gamma$ .<sup>23</sup>  $T_H1$  responses are critical in controlling mycobacterial pulmonary infections and downregulating  $T_H2$  cell activity.<sup>24-26</sup> The functioning of the pulmonary immune response depends not only on the local cytokine milieu but also on the regulation of leukocyte infiltration, which is mediated by the interaction of chemokines and their corresponding receptors. The chemokine receptor CXCR3 is strongly expressed on activated  $T_H1$  and T cytokine type 1 (Tc1) cells.<sup>27-29</sup> CXCR3 mediates chemotaxis of lymphocytes in response to its ligands, such as chemokine (C-X-C motif) ligand (CXCL)9/MIG, CXCL10/IP-10, and CXCL11/ITAC,<sup>30</sup> which can be produced by various cell types including endothelium, macrophages, and neutrophils, and the expression of each is induced by IFN- $\gamma$ .<sup>29,31-34</sup> CXCR3 expression on infiltrated immune cells may reflect local production of IFN- $\gamma$ . These cells, through the production of IFN- $\gamma$ , can further regulate CXCL chemokine production, thus establishing a mechanism for maintenance of a normal type 1 immune response in the lung. The current study shows the mRNA expression for IFN- $\gamma$ , CXCL chemokines MIG, IP-10, and ITAC were significantly decreased in the lungs of infant monkeys with perinatal exposure to ETS. The number of immune cells expressing CXCR3 in the subepithelium was also significantly decreased by exposure to ETS. Although we did not observe such clear effects on  $T_H1$ -associated chemokines in the postnatal ETS monkeys, the direction of changes for these

chemokines was similar to that in perinatal ETS monkeys in comparison with FA controls. These observations strongly suggest ETS exposure in early life alters the pulmonary microenvironment by attenuating type 1 immune responses in the lung. Many of the  $T_H1$  cytokines and chemokines measured in this study play important roles during viral infections, and adequate upregulation of these cytokines is crucial to resolve infection. Consequently, impaired ability to activate a  $T_H1$ -dominant antiviral defense response as a result of perinatal/postnatal ETS exposure could enhance the risk of children exposed to passive cigarette smoke to viral infections, such as respiratory syncytial virus.

The mechanisms by which ETS diminishes  $T_H1$  responses in the lung remain obscure. It has been suggested that several events in an immune response influence the ensuing  $T_H1/T_H2$  balance.<sup>35</sup> Candidate molecules such as IL-12 and IL-10 have been identified.<sup>35-37</sup> Bioactive IL-12p70 is a powerful promoter of  $T_H1$ -cell development by induction of IFN- $\gamma$  and suppression of IL-4 production.<sup>38,39</sup> IL-10 is the physiologically most relevant inhibitor of IL-12 production and the  $T_H1$  immune responses.<sup>40</sup> IL-10 has also been shown to inhibit production of CXC chemokines.<sup>41</sup> In our study, we demonstrated that both perinatal and postnatal ETS monkeys had suppressed IL-12 production, which is consistent with a previous report that sidestream cigarette smoke exposure leads to a significant decrease in pulmonary IL-12 and IFN- $\gamma$  levels in a neonatal mouse model.<sup>42</sup> It is possible that the observed decrease in IFN- $\gamma$  and type 1 chemokine expression in the current study might be attributed to a decreased IL-12 concentration that could not be related to inhibitory effects of IL-10, because both cytokine levels were reduced in both ETS treatment groups. Other mediators, such as prostaglandin  $E_2$ , might have an inhibitory effect on IL-12 production by exposure to ETS.<sup>43</sup> The effects of cigarette smoke on IL-10 production are contradictory: both increased and decreased IL-10 production have been described in response to cigarette smoke or its components in isolated T cells from peripheral blood,<sup>44</sup> in serum samples,<sup>45</sup> and in bronchial alveolar macrophages.<sup>46</sup> Moreover, data on lung IL-10 measurements in infants exposed to ETS are limited.

We previously described both perinatal and postnatal ETS groups having an increase in IL-10 mRNA expression in PBMCs during the first year of life,<sup>19</sup> whereas a decrease in IL-10 concentration was noted in the lung tissue from these infant monkeys exposed to ETS. The exact mechanisms for these findings are unclear at this moment. The systemic and mucosal components of the immune system are interrelated but develop independently and at different ages.<sup>1</sup> Although IL-10 is produced in high amounts by inflammatory cells, it can also be produced in substantial amounts by structural cells like epithelial cells.<sup>47</sup> Therefore, ETS may differentially affect IL-10 expression in the airway by regulating its expression in epithelial cells.

The transcription factors T-bet and GATA-3 are also crucial in determining the type of immune response. T-bet is an important activator of the IFN- $\gamma$  gene,<sup>11,48,49</sup> whereas GATA-3 is known to initiate  $T_H2$  cytokine transcription,<sup>50-52</sup> thus, differential expression of these factors is likely to be important in the acquisition of the  $T_H1$  or  $T_H2$  phenotypes, respectively. The ability of T-bet to regulate expression of type 1 chemokines, such as IP-10 and MIG, was demonstrated by a recent study.<sup>53</sup> As reviewed by Robinson and Lloyd,<sup>11</sup> expression of t-bet or GATA-3 not only determines  $T_H$  differentiation but also can actually override the influence of exogenous polarizing stimuli or previous polarization. In the current study, significant positive correlations between

mRNA expression of T-bet and IFN- $\gamma$  ( $r = 0.794$ ;  $P < .01$ ), IL-2 ( $r = 0.638$ ;  $P < .05$ ) and CXCR3 ( $r = 0.808$ ;  $P < .01$ ;  $n = 10$ ; data not shown) were present in monkeys exposed to ETS. The positive correlations between T-bet and  $T_H1$ -related chemokines IP-10 ( $r = 0.523$ ;  $P = .121$ ), MIG ( $r = 0.578$ ;  $P = .08$ ), and ITAC ( $r = 0.55$ ;  $P = .1$ ) were marginal and not statistically significant. These findings suggest the possible regulatory effects of ETS on the expression of T-bet and its correlation with the cytokines and chemokine expression in lung tissue. Further research is needed in this regard.

We performed bronchoalveolar lavage for each animal. The cell differential from bronchoalveolar lavage did not show statistically significant differences between the filtered air group and each of the groups exposed to ETS (data not shown). Furthermore, we examined the tissue localization of the CXCR3<sup>+</sup> immune cells in peripheral airways. Our data revealed that the decreased expression of the chemokine receptor in ETS-exposed monkeys was in the subepithelium area but not in the parenchyma, suggesting that the immune response at different microenvironments within the airway wall may be differentially affected by ETS exposure.

The association between exposure to ETS in childhood and risk of atopic sensitization has been extensively studied. At this point, we do not know whether these monkeys will develop asthma in the long run. However, we observed an elevated baseline total IgE level in serum samples in perinatal ETS-exposed animals compared with FA controls (data not shown). These ETS-exposed animals did not receive any allergen challenge because they were housed in only a pure chamber with ETS exposure. The increased total IgE in perinatal ETS-exposed monkeys not only suggests a reflection of susceptibility to allergen because of ETS exposure during the perinatal period but also serves as an indicator for the development of atopic phenotype in these infant monkeys.

In conclusion, our results indicate that ETS exposure during early life promotes a local  $T_H2$  immunity by impairing normal pulmonary  $T_H1$  immune maturation. Specifically, exposure to ETS beginning *in utero* resulted in a much greater alteration of  $T_H1$  factors than exposure beginning during the postnatal period. We speculate that ETS might be able to modulate the expression of transcription factor T-bet, which may be involved in the decrease in  $T_H1$  cytokine and chemokine expression as well as decreased expression of CXCR3-positive immune cells in the lungs. Moreover, the observed pulmonary immune phenotype in the current study was consistent with the systemic immune response that we reported previously. We also noticed a significant correlation between the mRNA expression of IFN- $\gamma$  in PBMCs and lung tissue ( $r = 0.628$ ;  $P = .012$ ;  $n = 15$ ), which suggests peripheral blood IFN- $\gamma$  could serve as a predictor of ETS effects on the phenotype of the developing immune system. These results provide new evidence to understand better the pathogenesis of ETS in the development of asthma or respiratory infectious diseases in children.

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**Clinical implications:  $T_H1$  responses are crucial in controlling mycobacterial pulmonary infections and downregulating  $T_H2$  activity. An impaired  $T_H1$  immune response may predispose infants to the development of allergic and infectious respiratory diseases.**

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## METHODS

### Reagents

The following reagents were purchased: RNA isolation kit, Omniscript reverse transcriptase, and QuantiTect SYBR Green PCR Kit from Qiagen (Valencia, Calif); anti-CXCR3 mAb from BD Biosciences (Franklin Lakes, NJ); AR-10 antigen retrieval solution from BioGenex (San Ramon, Calif); protein block and diaminobenzidine substrate kit from Dako (Carpinteria, Calif); EnVision/goat antimouse IgG (Dako, Glostrup, Denmark); horseradish peroxidase from Dako (Glostrup, Denmark); hematoxylin from Vector Laboratories Inc (Burlingame, Calif); and IL-10 and IL-12p70 ELISA kits from BD Biosciences (San Jose, Calif).

### Animal and exposure to ETS

Fifteen pregnant rhesus monkeys (*Macaca mulatta*) from the animal colony at the California National Primate Research Center (Davis, Calif) were obtained, and their infants were assigned to 3 different treatment groups: (1) exposed to FA ( $n = 5$ ) from gestational day 50 to 13 months of age, (2) exposed to ETS from gestation day 50 to 13 months of age (perinatal ETS;  $n = 5$ ), and (3) exposed to ETS from 6 months of age to 13 months of age (postnatal ETS;  $n = 5$ ). The dams were selected on the basis of a history of successful pregnancies. Treatment groups included both male and female infants. All studies involving animals conformed to the regulations of the American Association for Accreditation of Laboratory Animal Care standard. Aged and diluted sidestream smoke was used as a surrogate for ETS and produced in a smoking apparatus built in our laboratory, as described previously.<sup>E1</sup> 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/m<sup>3</sup>. Both perinatal ETS and postnatal ETS groups received ETS exposure under the same conditions (for detailed exposure conditions, see Table E1).

### Necropsy and tissue sampling

An overdose of pentobarbital (60 mg/kg) was administered by intravenous injection to each infant the day after the termination of ETS exposure. After exsanguination, the thorax was opened by midline incision and the entire mediastinal contents removed. The superior and inferior lobes of the right lung were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The left lung (superior and inferior lobes) and the right middle lobe were fixed by airway infusion with 4% paraformaldehyde at a hydrostatic pressure of 30 cm for 3 hours and stored in fixative. Tissue samples from each fixed lung lobe were embedded in paraffin, sectioned, and stained to examine the airways and parenchyma.

### Quantitative real-time PCR

Total RNA was isolated from lung tissue of right inferior lobes using a high pure RNA isolation kit, and cDNA synthesis was performed as described by Vogel et al.<sup>E2</sup> Briefly, DNA-free total RNA (1.0  $\mu\text{g}$ ) was reverse-transcribed by using 4 units of Omniscript reverse transcriptase and 1 mg oligo(dT) in a final volume of 40  $\mu\text{L}$ . Primers of IFN- $\gamma$ , IL-2, IL-4, IL-13, CXCR3, CCR5, IP-10, ITAC, MIG, CCR3, CCR4, eotaxin, TARC, t-bet, GATA-3, and GAPDH were selected from published sources and described in Table E2. Quantitative detection of GAPDH and these primers was performed with a LightCycler Instrument (Roche Diagnostics, Indianapolis, Ind) using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, Calif) according to the manufacturer's instructions. PCR amplification was performed in a total volume of 20  $\mu\text{L}$ , containing 2  $\mu\text{L}$  cDNA, 10  $\mu\text{L}$  QuantiTect SYBR Green PCR Master Mix, and 0.2  $\mu\text{mol/L}$  each primer. The PCR cycling conditions were  $95^{\circ}\text{C}$  for 15 minutes followed by 40 cycles of  $94^{\circ}\text{C}$  for 15 seconds,  $60^{\circ}\text{C}$  for 20 seconds, and  $72^{\circ}\text{C}$  for 10 seconds. Detection of the fluorescent product was performed at the end of the  $72^{\circ}\text{C}$  extension period. Negative controls were run concomitantly to confirm that the samples were not cross-contaminated. A sample with DNase-free and RNase-free water instead of RNA was concomitantly ex-

amined for each of the reaction units described. To confirm the amplification specificity, the PCR products were subjected to melting curve analysis. All PCR assays were performed in duplicate or triplicate. The intra-assay variability was  $<7\%$ . Quantification data were analyzed with the LightCycler analysis software according to the manufacturer's instructions. The comparative delta Ct method (Perkin Elmer User Bulletin no. 2, 1997; Perkin Elmer, Emeryville, Calif) was used for quantitation of mRNA using GAPDH mRNA as the endogenous reference. Results are presented as ratios of target mRNA to GAPDH mRNA.

### Immunohistochemical staining of CXCR3 in the airways

Chemokine CXCR3 receptor 3 in both bronchi and respiratory bronchioles was stained with anti-CXCR3 made in the mouse by using the following immunohistochemical procedure. Paraffin-embedded sections of left inferior lobes were taken through xylene and ethanol to remove the paraffin and treated with AR-10 antigen retrieval solution. Endogenous peroxidase was blocked with 3%  $\text{H}_2\text{O}_2$ . Slides were washed in PBS, and protein block was applied as a nonspecific blocking step. Sections were incubated in anti-CXCR3 antibody for 1 hour at room temperature, and then washed in PBS. Sections were subsequently incubated in EnVision/goat antimouse IgG, horseradish peroxidase, and DAB substrate according to the manufacturer's directions. Sections were counterstained with hematoxylin. Sections of spleen known to be positive for this antigen were used as positive controls, and primary antibody was substituted with normal serum as a negative control.

### Semiquantitative assessments of CXCR3-positive cells

The infiltrating mononuclear cells that stained positively for CXCR3 were determined for both the subepithelium and the parenchyma. Light-microscopic analysis was performed for quantification of CXCR3-positive cells. In the subepithelium, positive cells were counted in the area 100  $\mu\text{m}$  beneath the epithelial basement membrane in selected nonoverlapping high-power fields until the entire available area was covered. For the lung parenchyma, as many as 40 fields of alveolar tissues were sampled. Field selection was based on a random start over parenchymal tissues and followed a systematic zigzag pattern to sample nonoverlapping fields of parenchymal tissues. The final result, expressed as the number of positive cells per square micrometer, was calculated as the average of all the cellular counts performed on each slide for each anatomical region for each animal.

### IL-10 and IL-12p70 detection by ELISA

The concentrations of monkey IL-10 and IL-12p70 in the lung tissue homogenate of the right inferior lobes were determined by ELISA kits according to the manufacturer's protocols.

### Statistical analysis

Statistical analyses were performed by using Statview statistical software (SAS Institute, Cary, NC). All data were presented as means  $\pm$  SEMs. Comparisons among ETS-exposed and FA-exposed groups were made by 1-way ANOVA followed by pairwise comparisons with *post hoc* Fisher protected least significant test. A value of  $P < .05$  was considered significantly different.

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- E2. Vogel CF, Zhao Y, Wong P, Young NF, Matsumura F. The use of c-src knockout mice for the identification of the main toxic signaling pathway of TCDD to induce wasting syndrome. *J Biochem Mol Toxicol* 2003;17:305-15.

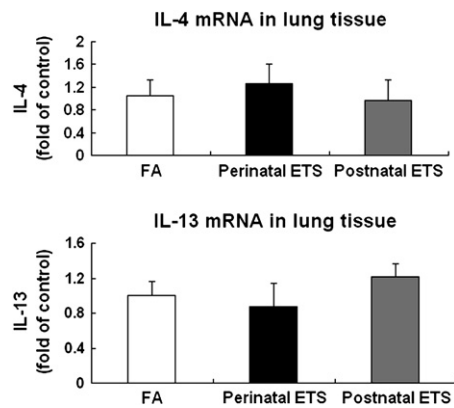


**FIG E1.** Relative levels of expression of mRNA for IL-4 and IL-13 in lung tissue from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. The data are expressed as the means  $\pm$  SEMs ( $n = 5$ ).

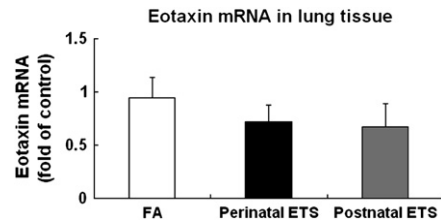


**FIG E2.** Relative levels of expression of mRNA for  $T_H2$  chemokine eotaxin in lung tissue from FA-exposed, perinatal ETS-exposed, or postnatal ETS-exposed infant monkeys. The data are expressed as the means  $\pm$  SEMs ( $n = 5$ ).

**TABLE E1.** ETS exposure conditions

|                                                  |             |
|--------------------------------------------------|-------------|
| Total suspended particulate (mg/m <sup>3</sup> ) | 0.97 ± 0.13 |
| Carbon monoxide (ppm)                            | 5.3 ± 0.8   |
| Nicotine (µg/m <sup>3</sup> )                    | 259 ± 62    |
| Temperature (°F)                                 | 77 ± 4      |
| Relative humidity (%)                            | 39 ± 10     |

All values are means ± SDs. Exposure conditions were monitored daily.

**TABLE E2.** Cytokine, chemokine, chemokine receptor, and transcription factors primer sequence

| Gene          | Sequence (5'-3')                  |                                        |
|---------------|-----------------------------------|----------------------------------------|
|               | Forward primer                    | Reverse primer                         |
| GAPDH         | GCA CCA CCA ACT GCT TAG CAC       | TCT TCT GGG TGG CAG TGA TG             |
| IFN- $\gamma$ | GAA AAG CTG ACC AAT TAT TCG GTA A | AGC CAT CAC TTG GAT GAG TTC A          |
| IL-2          | AAG AAT CCC AAA CTC ACC AGG AT    | TCT AGA CAC TGA AGA TGT TTC AAT TCT G  |
| IL-4          | CAG CCT CAC AGA GCA GAA GAC TC    | TTG TGT TCT TGG AGG CAG CA             |
| IL-13         | GGA TTC TGC CCG CAC AAG           | AAA CTG GGC CAC CTC GAT T              |
| CXCR3         | GCA ACC TCG GCG TCA TTT           | CAA CCA CAA GCA CCA AAG CA             |
| CCR5          | CCG ACG AAG GCA TAG ATG AT        | TCT CTT CTG GGC TCC CTA CA             |
| CCR3          | TAA AAC CCC GAG AGG ACC TT        | GAG GCT CCG AAT TAT GAC CA             |
| CCR4          | AGC CCA CCA AGT ACA TCC AG        | GTG GTG GTT CTG GTC CTG TT             |
| IP-10         | TTG ATG GCC TTA GAT TCT GGA       | TTG CTG CCT TGT CTT TCT GA             |
| MIG           | CAG ATT CAG CAG ATG TGA AGG AA    | ACG TTG AGA TTT TCT AAC TTT CAG AAC TT |
| ITAC          | TGG GAT TTA GGC ATC GTT GT        | AGA GGA CGC TGT CTT TGC AT             |
| Eotaxin       | TAT CCT TGG CCA GTT TGG TC        | CAA CAC TTC TGT GGC TGC TG             |
| TARC          | AAC AGA TGG CCT TGT TCT GG        | CTT CTC TGC AGC ACA TCC AT             |
| t-bet         | CCG TGA CTG CCT ACC AGA AT        | ATC TCC CCC AAG GAA TTG AC             |
| GATA-3        | TAG CAG TGA CCA TGC TGG AG        | CCA GAC CAG AAA CCG AAA AA             |