

Original article

Maternal smoking in pregnancy alters neonatal cytokine responses

Background: Maternal cigarette smoking in pregnancy is an important, common and avoidable exposure that has been linked with elevated cord blood (CB) immunoglobulin E levels and subsequent asthma and allergic disease in childhood. Despite this, there is still very little information about the immunological effects of maternal smoking on the fetus.

Methods: This aim of this study was to compare cord blood mononuclear cell (CBMC) cytokine responses to allergens [ovalbumin (OVA) or house dust mite (HDM)] and mitogens [concanavalin A (ConA) or phytohemagglutinin (PHA)] in neonates whose mothers smoked throughout pregnancy ($n = 17$) with responses of neonates whose mothers never smoked ($n = 40$). Cell cultures were stimulated for 24 h and supernatants collected for cytokine detection by enzyme-linked immunosorbent assay [interleukin (IL)-13, IL-6, interferon (IFN) γ and IL-10]. Cell pellets were also collected for cytokine mRNA detection (IL-5, IL-9, IFN γ).

Results: Maternal smoking in pregnancy was associated with significantly higher neonatal T helper type 2 (IL-13 protein) responses to both HDM ($P = 0.01$) and OVA ($P = 0.035$). These effects remained statistically significant after allowing for confounding factors, including the effects of maternal atopy. Similar trends were also seen for IL-9mRNA, IL-5mRNA and IL-6 responses, although these were not statistically significant. Although IFN γ mRNA responses to PHA ($P = 0.015$) and ConA ($P = 0.025$) were lower if mothers smoked in pregnancy, there were no differences in neonatal (Th1) IFN γ protein responses to allergens or mitogens.

Conclusions: These findings indicate that maternal cigarette smoking can modify aspects of fetal immune function and highlight the need for further studies in this area.

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Escalating rates of asthma and allergic diseases appear to be the result of complex environmental changes and predisposing genetic factors. There is now strong evidence that environmental exposures during pregnancy can have profound effects during critical periods of fetal development, and that this may dictate patterns of vulnerability to insults in the postnatal period. Maternal cigarette smoking is a common, avoidable exposure that has been associated with a significant increase in airways pathology in childhood. While there have been extensive studies on the effects of cigarette smoking on neonatal lung function and development (1–5), there is very limited information about the effects on the developing neonatal immune responses. It is now well recognized that the neonate is capable of responding to allergens (although the significance of these *in vitro* responses is unclear) and that early dysregulation of these responses is associated with the development of allergy in infancy. Alternations in both Th1 (6–10) and T helper type 2 (Th2) (11–13) responses to allergens during the neonatal period have been associated with both atopic risk and subsequent

disease (as reviewed in the Discussion). Maternal cigarette smoke is an important common exposure during this critical period which needs to be investigated for potential effects on neonatal cytokine responses to allergens and other stimuli. Earlier studies (14) noted that parental smoking is associated with higher cord blood (CB) immunoglobulin E (IgE) levels, suggesting immunological effects of smoke exposure in the antenatal period. More recently, Devereux et al. observed that maternal smoking is associated with stronger neonatal lymphoproliferation (15), but the effects on cytokine profiles and allergen-specific responses are still unknown. A number of studies have linked parental smoking with markers of atopy in children (16, 17), including serum IgE levels (18–21) eosinophilia (20, 22), and positive skin prick tests (SPTs) (23). These observations suggest immunological effects in addition to airway irritation and early effects on lung mechanics. However, there is currently virtually no other information about the mechanisms of these effects and the underlying immunological changes in infants of smoking mothers. With the continuing high rates

(25–30%) of smoking among women of child bearing age (24) there is an urgent need to investigate the potential role in the development of Th2 associated allergic disease. The purpose of this analysis was to further examine the association between maternal smoking in pregnancy and neonatal cytokine responses.

Materials and methods

Study subjects and samples collected

A random sample of women ($n = 57$) booked for elective caesarean sections at King Edward Memorial Hospital and St John of God Hospital were recruited into the study, including women ($n = 17$) who smoked throughout pregnancy and women ($n = 40$) who did not smoke at any stage of pregnancy. Elective caesarean deliveries were selected to minimize the confounding effects of labor on delivery method (which have been shown previously to influence CB cytokine measures) (25). Maternal health interview questionnaires were completed with a medical practitioner, to obtain data on allergy history and smoking history, including past or present smoking, the number of cigarettes smoked per day, and at what stages in pregnancy. Cotinine levels were not measured. Women were ineligible for the study if they had medical problems, complicated pregnancies, and were subsequently excluded if their delivery was preterm (< 36 weeks gestation) or if there were any maternal or infant complications at birth. Maternal allergy was also assessed by SPT to common allergens [house dust mite (HDM), grasses, moulds, cat, dog, feathers and cockroach (Hollister-Stier Laboratories, Spokane, WA)]. A wheal size of ≥ 3 mm above the negative control was considered positive. Maternal blood was also collected for total serum IgE levels. Women were defined as allergic if they had a history of doctor diagnosed allergic rhinitis and/or asthma and one or more positive SPT. Nonallergic women had no history of disease and no positive SPT. By these criteria, 64.7% (11 of 17) of the smokers and 57.5% of the nonsmokers (23 of 40) were allergic.

Informed consent was obtained from all mothers, and the study was approved by the Princess Margaret Hospital and the King Edward Memorial Hospital Ethics Committee.

Cord blood collection and neonatal immune responses

Cord blood samples were collected from the placental vessels into heparinized Roswell Park Memorial Institute (RPMI; Life Technology, Paisley, UK) tissue culture medium as previously described (26). Mononuclear cells [cord blood mononuclear cell (CBMC)] were isolated from CB samples using Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient centrifugation and cryopreserved for subsequent batch analysis. It has been shown previously that cryopreservation and thawing does not distort MC cellular immune responses (27–29).

For lymphoproliferation, CBMC were cultured in triplicate for 7 days in microwells at 10^9 cells/l in serum-free medium, either alone or in the presence of HDM allergen extract 10 $\mu\text{g/ml}$ or ovalbumin (OVA) 100 $\mu\text{g/ml}$. Data were expressed as the difference in disintegrations per min (dpm) between the stimulated culture and background (unstimulated) control cultures. Responses that were greater than twice the background counts and more than 1000 dpm above the background counts were classed as positive.

For cytokine analysis, CBMC were cultured for 24 h in medium alone, or supplemented with optimal stimulating concentrations of HDM 10 $\mu\text{g/ml}$, OVA 100 $\mu\text{g/ml}$, concanavalin A (ConA) 5 $\mu\text{g/ml}$, or phytohemagglutinin (PHA) 1 $\mu\text{g/ml}$. Supernatants were assayed for the presence of interleukin (IL)-13, IL-6, IL-10 and interferon

(IFN) γ employing commercial enzyme-linked immunosorbent assay kits (CLB Laboratories, Amsterdam, The Netherlands). For cytokines produced at lower levels (IL-5, IL-9 and IFN γ) together with β -actin, specific mRNA was measured in cell pellets by standardized semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) as described in (30). The RNA was extracted from cell pellets using RNeasy B, and cDNA was transcribed using oligo (dT)15 and avian myeloblastosis virus reverse transcriptase in the presence of ribonuclease inhibitor. The PCR reactions employed primers specific for IL-4, IL-5, IL-9, IFN γ and β -actin plus TthPlus DNA polymerase, and were performed in a programmable thermocycler. The intensity of each test sample was expressed as a ratio relative to that of its respective β -actin control.

Statistical analysis

Relationships between categorical variables were analysed using Pearson's chi-square test. All continuous variables (cytokine protein, mRNA expression, antibody concentrations) were $\log_{10}$ transformed to correct for skewness and kurtosis of the variable (confirmed for each data set). Student's t -tests were used to compare maternal smoking status groups on the $\log_{10}$ transformed continuous variables. Where the relationship between maternal smoking status and a continuous variable was potentially confounded by maternal and/or family history of allergy, standard multiple linear regression was used to examine the relationship between maternal smoking status (as a binary predictor variable) and the immunological outcome after controlling for the two confounding variables. A P value < 0.05 was considered statistically significant for all analyses. The SPSS (version 10.0 for Macintosh) were used for data analysis.

Results

Maternal characteristics

The proportion of atopic women was slightly higher in the smoking group (64.7%; 11 of 17) compared with that of the nonsmokers (57.5%; 23 of 40), however this was not significant ($P = 0.61$). As anticipated, maternal allergic disease was associated with elevated maternal IgE levels ($P = 0.0001$). Mothers who smoked also had significantly higher IgE levels ($B = 0.321$, 95% CI 0.088, 0.555; $P = 0.008$) after allowing for the influence of maternal history of allergy by linear regression. Smokers claimed to consume between 5 and 35 cigarettes/day throughout their pregnancy. The outcomes were also compared on the number of cigarettes smoked per day.

The relationship between maternal smoking and lymphoproliferation

In this study there was no relationship between maternal smoking and lymphoproliferation to allergens or mitogens (not shown).

The relationship between maternal smoking and neonatal Th2 responses

In general maternal smoking was associated with increased neonatal Th2 (IL-5, IL-9, IL-13) responses to allergens.

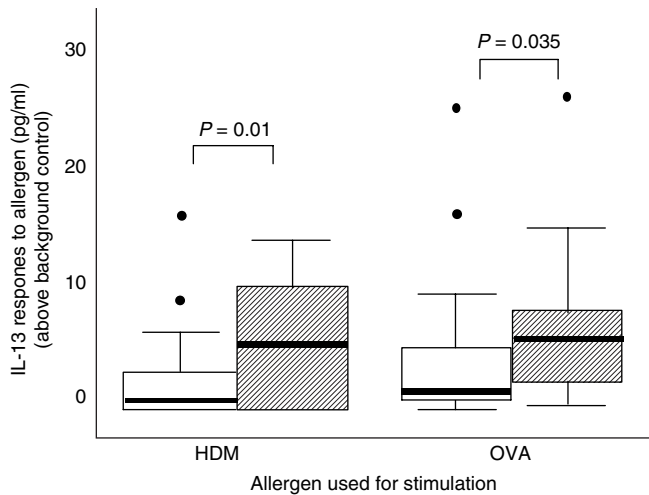


Figure 1. Comparison of allergen-specific interleukin (IL)-13 responses by neonates of smokers and nonsmokers. Supernatant IL-13 levels (after 24 h) in response to house dust mite and ovalbumin stimulation are shown for infants of nonsmokers (clear bars) and smokers (shaded bars). Levels above unstimulated control cultures are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. Groups were compared using parametric independent Student's *t*-tests (significance levels are as shown).

Specifically, maternal smoking was associated with significantly higher IL-13 responses (Fig. 1) to both HDM ($B = 0.349$, 95% CI 0.086, 0.612; $P = 0.01$) and OVA ($B = 0.284$, 95% CI 0.021, 0.546; $P = 0.035$) after allowing for the influence of maternal and family history of allergy by linear regression. However, this relationship was not seen in response to mitogen stimulation.

There was also a trend for higher IL-5 responses in neonates whose mothers smoked, particularly for OVA (Fig. 2) although this was not statistically significant ($B = 2.884$, 95% CI -0.693 , 6.462; $P = 0.112$). However there was a positive relationship ($r = 0.34$, $P = 0.015$) between the number of cigarettes smoked (/day) and IL-5 mRNA responses to OVA (but not HDM).

There was also a positive relationship between IL-9 responses to HDM and maternal smoking in pregnancy (not shown) however this was no longer statistically significant ($B = 0.175$, 95% CI -0.081 , 0.431; $P = 0.175$) after the effects of family history were taken into account using regression modelling.

The relationship between maternal smoking and neonatal IL-6 and IL-10 responses

Maternal smoking during pregnancy was associated with a tendency for weaker neonatal IL-10 and IL-6 responses (to HDM), but this also, did not achieve statistical significance (not shown).

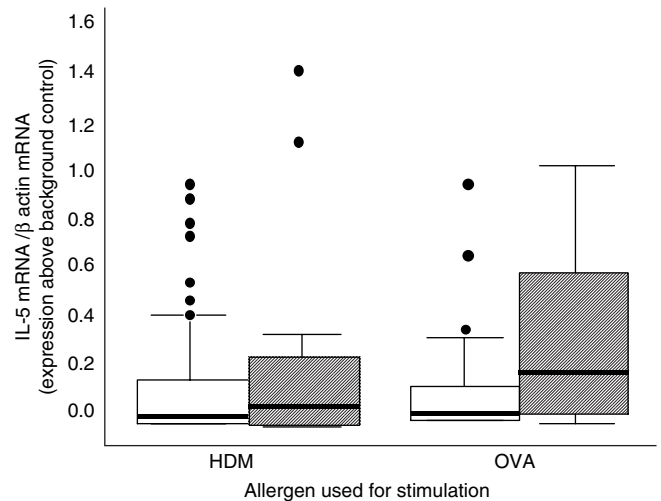


Figure 2. Comparison of allergen-specific interleukin (IL)-5 responses by neonates of smokers and nonsmokers. CBMC IL-5 mRNA levels (after 24 h) in response to house dust mite and ovalbumin stimulation are shown for infants of nonsmokers (clear bars) and smokers (shaded bars). Levels are expressed as the ratio of cytokine/ β actin specific mRNA, and responses are displayed as the levels above unstimulated control cultures (median, 5, 25, 75, 95 percentile ranges and outlying values). Groups were compared using parametric independent Student's *t*-tests.

The relationship between maternal smoking and neonatal Th1 responses

Although babies born to smoking mothers had significantly lower Th1 IFN γ mRNA responses (not shown) to both PHA ($B = -0.304$, 95% CI -0.549 , -0.060 ; $P = 0.015$) and ConA ($B = -3.294$, 95% CI -6.149 , -0.438 ; $P = 0.025$) there was no significant difference in IFN γ responses levels in these cultures. The Th1 IFN γ responses to allergens were largely below detection by both methods, and not altered by smoking.

Discussion

To our knowledge, this is the first study to examine the relationship between maternal smoking in pregnancy and neonatal cytokine responses to various stimuli, including allergens. The findings suggest that *in vitro* CBMC-cytokine responses are influenced by this important avoidable risk factor for asthma and atopic disease.

Although the significance of perinatal immune responses is still unclear, a number of variations in neonatal immune function have been associated with both genetic risk of atopy (based on family history) and subsequent atopic disease (6, 8–10, 31–33). While neonatal T cell responses to allergens may not measure conventional T cell 'memory' (34), variations in responses may reflect individual differences in default response

patterns, and are of therefore of interest in understanding dysregulation of normal immune development during this critical early period. Furthermore, factors that potentially modify early immune responses are also of key interest. The findings of the present study support the likelihood that variation in neonatal immune function is not merely genetically determined, but may be influenced by antenatal exposures. This is in keeping with the observation that other exposures, such as infection *in utero*, may also modify neonatal immune responses (35).

Although the immunologic events leading to allergic disease are highly complex and multifactorial, there is accumulating evidence that disruptions in Th1/Th2 immune regulation in early life may be implicated in some way. In this study, we observed that neonatal Th2 responses (particularly IL-13) were significantly stronger if mothers smoked in pregnancy. This apparent pro-Th2 effect is in keeping with previous observations that peripheral blood mononuclear cells from smokers secrete significantly more Th2 cytokines (IL-4) *in vitro* compared with nonsmokers (36). We did not see any significant effects of maternal smoking on neonatal Th1 IFN γ responses, when measured as cytokine protein. As protein levels are more likely to reflect functional effects, the relevance of observed effects of smoking on IFN γ mRNA expression is unclear. In the perinatal period, a relative dominance of Th2 responses is normal, and appears to result from both the hormonal effects of pregnancy (30) and an inherent immaturity of Th1 function during this period of life (26). It is still not clear how variations in neonatal Th2 responses relate to the subsequent development of allergic disease. While a number of recent studies have linked stronger perinatal allergen-specific Th2 responses (particularly IL-13 responses) with atopic risk or subsequent allergic disease (11–13, 37) the significance of this is still uncertain. Our observations here suggest that maternal smoking in pregnancy may directly influence aspects of these perinatal immune responses. The apparent effects on Th2 responses may reflect the potential of maternal smoking to disrupt complex normal immune regulation during the antenatal period, highlighting the need for further studies to assess the effects on subsequent immune development. We speculate that *in utero* immune effects of smoking could compound the inflammatory effects of postnatal exposure to increase the risk of abnormal postnatal immune development. Thus, while maternal smoking has already been associated with increased respiratory morbidity and reduced lung function in infants and children, these findings suggest that maternal cigarette smoking during pregnancy may also modify fetal immune development. The nature of these interactions needs to be more fully investigated.

Most previous studies to examine the potential relationship between maternal cigarette smoking during pregnancy and immune development have explored effects on neonatal CB IgE levels, childhood serum IgE

levels, and allergen skin test reactivity (38). As previously stated Magnusson et al. (14) found higher CB IgE levels in children of mothers, but not fathers, who smoked. However, most other studies of CB IgE and maternal smoking have not confirmed this (38). Some studies suggest an association between *in utero* smoking exposure and postnatal serum IgE levels (18, 20) or SPT (16, 39), but these studies were unable to implicate *in utero* smoking exposure as distinct from postnatal exposure. Furthermore, other studies have yielded conflicting relationships between maternal smoking and both serum IgE (40, 41) and skin prick testing (42, 43). More recently however, Gilliland et al. (44) observed that smoking may exert an important *in utero* effect on the fetal immune system. In a cohort of 5762 school-aged children (44), they demonstrated that *in utero* exposure to maternal smoking without childhood passive smoke exposure was associated with an increased prevalence of physician-diagnosed asthma (OR 1.8, 95% CI 1.1, 2.9), a lifetime history of wheezing (OR 1.8, 95% CI 1.2, 2.6) and persistent wheezing (OR 3.1, 95% CI 1.6, 6.1). Current or previous postnatal passive smoke exposure was not associated with an increased prevalence of childhood asthma, but was consistently associated with childhood wheezing. In most situations, infants exposed to maternal smoking during pregnancy are also likely to be reared with passive postnatal smoke exposure, making it difficult to distinguish the separate effects of *in utero* and postnatal environmental exposures to tobacco smoke. Passive smoking in the early postnatal period may have immunological effects at mucosal surfaces, where inflammation may influence local allergen processing and events in regional lymph nodes. As the main point of contact with the environment, the mucosal immune system also has enormous potential to influence the development of systemic immune responses as well as local disease expression. Thus, further studies are also needed to examine the relationship between cigarette smoke exposure and immune events at mucosal surfaces.

Our data were based on the self-reported smoking behavior of women. This is a potential limitation of the study, which would have been more robust if we had prospectively measured serum cotinine concentrations in order to validate the reported smoking histories. The main source of inaccuracy is that smokers are likely to under-report cigarette consumption (rather than over-report it), which is unlikely to diminish the effects in this study. It is less likely, but possible that women who claimed to be nonsmokers smoked cigarettes. Further studies are required to further assess relationships between smoking and *in utero* immune development with more accurate measures of cigarette smoke exposure.

In conclusion, with the high rates of maternal smoking in pregnancy, it is imperative to determine how cigarette smoke influences the fetus during pregnancy. The findings

of this study indicate that maternal cigarette smoking can significantly alter aspects of fetal immune function, and hence can potentially influence both quantitative and qualitative aspects of the immune responses to initial challenges with environmental allergens during the immediate postnatal period. Further studies are now needed to assess this relationship and the subsequent effects in more detail.

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