

Maternal reactivity to fetal alloantigens is related to newborn immune responses and subsequent allergic disease

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Summary

Background Maternal allergy confers stronger allergy risk (than paternal allergy) suggesting that maternal patterns of immune response can directly influence immune development in offspring. Women prone to allergic immune responses to allergens may also have altered immune responses to other antigens including fetal antigens.

Objective This exploratory study examines relationships between maternal immune responses to fetal antigens and the subsequent risk of allergy.

Methods Mononuclear cells (MNC) were collected from 36 mother–infant pairs to compare maternal (and fetal) cellular immune responses to alloantigens (fetal, maternal or unrelated donor [URD]), and allergens in allergic (18 pairs) and non-allergic (18 pairs) mothers. Thirty children had documented allergic outcomes at 6 years of age.

Results In this population, allergic outcomes in the offspring were associated more strongly with materno-fetal immune interactions than with a maternal family history of allergy. Specifically, allergic disease at 6 years of age was associated with significantly higher maternal responses to fetal alloantigens (lymphoproliferation, $P = 0.008$; IL-13, $P = 0.02$ and IFN- γ , $P = 0.015$), whereas associations with maternal allergy did not reach significance ($P = 0.07$). Fetal IFN- γ alloantigen responses were significantly correlated with the degree of human lymphocyte antigen (HLA) mismatch (maternal HLA class II antibodies) ($\tau = 0.3$, $P = 0.03$). The capacity of the fetus to produce IL-13 ($\tau = 0.4$, $P = 0.001$) and IL-10 ($\tau = 0.3$, $P = 0.029$) was directly related to the level of these cytokines produced by the mother in response to fetal antigens. Allergic mothers showed a non-significant trend for stronger lymphoproliferation to fetal alloantigens. The number of previous pregnancies (gravidity) was associated with stronger maternal responses to fetal alloantigens, as shown by lymphoproliferation (Kendall $\tau = 0.3$, $P = 0.04$) and IFN- γ ($\tau = 0.3$, $P = 0.04$) synthesis, but did not affect fetal responses to the various stimuli.

Conclusions Maternal responses to fetal antigens were related to fetal immune responses and subsequent allergy. This novel observation suggests that events at the materno-fetal interface have an important influence on early immune development and should be investigated further.

Keywords allergy, alloantigens, cord blood, cytokines, placenta, pregnancy

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Introduction

Interactions between the mother and fetus influence the patterns of immune responses in pregnancy and the developing fetus. As we have shown previously, altered cytokine responses at birth have significant implications for subsequent immunological development and allergic disease [1, 2]. There is also evidence indicating a direct influence of maternal atopy (rather than paternal allergy) on T helper cell type 1 (Th1) dysfunction at birth [3]. The recognized predisposition for allergic Type 2 T helper cell type 2 (Th2) responses in atopic

women may modify immune responses in pregnancy and alter fetal immune responses directly. While the Th2 responses in allergy are typically directed to environmental allergens, other immune responses also appear altered, including the capacity of antigen-presenting cells (APC) from atopic donors to evoke allogenic T cell responses [4]. Consequently, we hypothesized that atopic females produce distorted Th1/Th2 responses in response to fetal alloantigens, compared with non-allergic women. Such variation in immunological interactions at the materno-fetal interface clearly could significantly influence the Th1/Th2 polarity both during pregnancy and in the early postnatal period.

Fetal responses are clearly sensitive to the ambient cytokine environment of pregnancy, and we have shown previously that the first cellular responses (in fetal life) universally reflect the 'normal Th2-skew' of pregnancy [5]. There is also growing

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evidence that altered T cell cytokine responses in fetal and early postnatal life are associated with allergic disease in pre-schoolchildren (or 2-year-old children) [1]. Thus, alterations in the cytokine balance at birth have implications for subsequent development of Th2-biased reactivity to environmental allergens, and must be investigated as a major factor in the aetiology of allergic diseases. Understanding the regulation of cellular immune activity in the feto-placental unit may not only be central to successful pregnancy but also to the pathogenesis of many disease processes with an immune basis.

This exploratory study investigates potential influences on maternal cell-mediated immunity in pregnancy and how this might influence fetal immune responses and the risk of subsequent allergic disease. The specific aims were (a) to assess how maternal immune responses to fetal antigens influence fetal immune development and subsequent allergic disease, and (b) to determine how this is affected by maternal allergy, previous pregnancies and the level of human lymphocyte antigen (HLA) incompatibility.

Materials and methods

Overview

We used mononuclear cells (MNC) samples collected from mother–infant pairs to examine maternal immune responses to fetal antigens, and how this might be related to maternal allergy, HLA antibodies, previous pregnancies, neonatal immune responses, and the development of subsequent clinical allergy.

Ethical approval

Ethical approval for collection of these samples and the associated studies was granted by the Princess Margaret Hospital Research and Ethics Committee.

Study population

This population included 36 mother–infant pairs. Thirty of these had longitudinal clinical data available (allergic outcomes at 6 years of age). All were of Caucasian background. There were 18 non-allergic and 18 allergic mothers. Pregnancy history was obtained by a maternal interview questionnaire. Information about previous pregnancies including spontaneous abortions, terminations and changes in paternity was collected from both the mother and the hospital charts. By this method, there were no documented changes in paternity, although the limitations of these data are recognized. All babies in the study were delivered by elective caesarean section and none of these women was in active labour. This mode of delivery was chosen to exclude the effects of active labour on cellular immune function. The indications for caesarean section included breech presentation or unstable lie, cephalo-pelvic disproportion/large for gestational age baby, and previous maternal abdominal surgery or caesarean section. In 5/36 women, there were no clear medical indications for the operative delivery. Women with other complicating factors (such as fever or any other conditions likely to affect immune function) were excluded. Maternal

MNC were isolated from peripheral blood collected in the perinatal period (generally within 24 h after delivery), and neonatal MNC were isolated from umbilical cord blood (CB). In preliminary studies (not shown), we compared maternal responses at various stages of gestation (20, 30, 36 weeks) and the post-partum period (6 weeks) and were unable to demonstrate any consistent differences in responses to mitogens, allergens or alloantigens (although there was a tendency for reduced lymphoproliferative responses in late pregnancy compared with 6 weeks post-partum). For consistency and logistic reasons we elected to collect blood in the perinatal period. Maternal allergic disease was defined by a clinical history of doctor diagnosed asthma and/or allergic rhinitis, and a positive skin prick testing to common aeroallergens (house dust mite (HDM), grass pollens and animal dander). In the children, allergic disease was defined (at 6 years) by a history of doctor-diagnosed atopic dermatitis and/or asthma. Data were available for 30 children (13 of non-allergic mothers and 17 of allergic mothers) at 6 years. Children with possible rhinitis, mild eczema or urticaria were only included as 'allergic' if other features of allergic disease (including positive skin prick test (SPT)) were present. SPTs were performed according to standardized techniques. The weal size was measured as the mean diameter (of the longest and the mid-point orthogonal diameters). A test was regarded as positive if the weal diameter was greater than 3 mm. Standardized allergens for skin prick testing were all manufactured by Hollister Stier (Miles Inc., Pharmaceutical Division, Elkhart, USA). These included HDM (standardized mite *Dermatophagoides pteronyssinus*) 30 000 AU/mL, southern grass mix (1 : 20 w/v), rye grass (perennial *Lolium perenne*) (1 : 20 w/v), mould mix #4 (1 : 10 w/v), standardized cat pelt (10 000 AU/mL), whole egg (1 : 20 w/v), peanut mix (Runner, Virginia, Spanish) 1 : 10 w/v, whole cows milk (1 : 20 w/v), negative control (glycerine) and positive control (Histamine phosphate 10 mg/mL).

Samples

CB samples were collected from the placental vessels by venepuncture immediately after delivery. Maternal peripheral blood was collected by routine venesection as specified above. As described previously, [6] MNC were isolated using Lymphoprep (Nycomed Pharma, Oslo, Norway) density gradient centrifugation and cryopreserved for subsequent studies of *in vitro* immunological responsiveness. All samples were checked for viability on thawing. Long-term cryopreservation does not significantly alter the pattern of cellular function of cytokine profiles (our unpublished data).

Antigen-stimulated immune responses

Lymphoproliferation and cytokine responses were performed as described previously [6, 7]. Briefly, lymphoproliferation was assessed using neonatal and adult MNC in round bottom plates (in triplicate). MNC (2×10^5 in 200 μ L of AIM V (Gibco, Life Technology, Paisley, UK) serum-free medium) were cultured alone or with optimal stimulating doses of HDM at 10 μ g/mL (CSL, Parkville, Australia). These cultures were incubated at 37 °C in 5% CO₂ for 6 days (neonates) or 5 days (adults). DNA synthesis was measured as incorporation

of ^3H -thymidine, after pulse labelling 16–18 h prior to harvest. Data were expressed as δ dpm per culture, designating levels of incorporation above background (unstimulated) control cultures.

To assess cytokine responses to the same stimuli, MNC were cultured in duplicate (5×10^5 in 250 μL of AIM V) in 96-well round bottom plates with the same stimuli (as above) or phytohaemagglutinin (PHA) at 1 $\mu\text{g}/\text{mL}$ (HA-16, Murex, Dartford, Australia). These were incubated in a 37 °C 5% CO_2 incubator for 48 h. Supernatants were then collected and stored at –20 °C until cytokine analysis was performed.

Alloantigen responses (mixed lymphocyte reactions)

Mixed lymphocyte reactions (MLR) were employed to measure the maternal response to CB mononuclear cells (CBMC) and thus the paternal HLA contribution. Autologous cells were used as the negative control (eliciting no response) and pooled unrelated donors (URD, from seven individuals) used as a positive control. ‘Stimulator’ populations were CD3-depleted (using Dynabeads as per manufacturers instructions (DynaL A.S., Oslo, Norway)) and irradiated at 3000 rad (Gammacell 3000 Elan, MDS Nordion, Ontario, Canada) to inhibit proliferation by this population. The same methods were used to assess fetal responses to maternal alloantigens and URD.

‘Responder’ (untreated) populations were made up to a concentration of 1×10^6 cells/mL in 250 μL AIM-V plus 5% heat-inactivated AB serum (HI AB, Biowhittaker, Walkersville, MD, USA). Stimulator cells (at 0.5×10^6 cells/mL) were then added and resulting cultures incubated for 72 h at 37 °C and 5% CO_2 . Cytokine measurements were measured at 72 h and lymphoproliferation at 5 days. On a control plate, responder and stimulator cell populations were stimulated separately (in 200 μL at 1×10^6 cells/mL) with recombinant IL-2 to ensure that the CD3 depletion and irradiation process was successful: non-irradiated responders showed IL-2-stimulated proliferation, whereas irradiated stimulators did not proliferate in response to IL-2 treatment.

Cytokine assays

Cytokines in cell culture supernatants were quantified using a ‘sandwich-type’ ELISA technique. Matched antibody pairs, consisting of unlabelled capture antibody and biotinylated detection antibody, and recombinant protein standards for

IL-5, IL-10, IL-13 and IFN- γ were purchased from Pharmingen (Becton Dickinson, San Jose, CA, USA). Antibodies were used at an optimal concentration established for our laboratory as described previously [6]. The sensitivities of the ELISA systems were 4 pg/mL (IL-5), 30 pg/mL (IL-10), 5 pg/mL (IL-13) and 5 pg/mL (IFN- γ).

Human lymphocyte antigen antibody levels

Sera were screened for antibodies to class I and class II HLA antigens using the One Lambda LAT-M ELISA (One Lambda Inc., Canoga Park, CA, USA) according to the manufacturer’s instructions. Levels are expressed in arbitrary units.

Statistical analysis

All statistical analysis was performed using Statistical Package for Social Sciences (SPSS; version 11.0 for Macintosh). The majority of data was not normally distributed. Where this could be normalized, natural logarithmic transformations groups were compared using parametric student *t*-tests and data displayed as geometric mean (and standard deviations). Where the data were unable to be normalized with logarithmic transformation, non-parametric tests were used (either Wilcoxon’s signed ranks or Mann–Whitney *U*-test for two group comparisons of paired and unpaired data, respectively). These data are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. Correlations were assessed using Kendall’s τb in order to avoid the problems associated with ‘ties’ within the data (where a proportion of the variables of interest shared ‘zero’ values). A level of $P = 0.05$ was considered statistically significant for all analyses. Because of the exploratory nature of this study, we have elected not to apply the Bonferroni correction factor for multiple analyses, but openly acknowledge the possibility of a type 1 error. The purpose of this was to identify important potential relationships that need to be investigated in larger studies.

Results

Comparison of allergic and non-allergic mothers

The characteristics of the study population are shown in Table 1. As anticipated, allergic women had higher IgE levels

Table 1. Population characteristics

	Non-allergic mothers ($n = 18$)	Allergic mothers ($n = 18$)	
Age (years)	Mean = 30.6 (SD 6.1)	Mean = 27.6 (SD 5.1)	NS
Gestation (weeks)	Mean = 38.9 (SD 1.09)	Mean = 38.5 (SD 0.93)	NS
Number of previous pregnancies	Mean = 2.33 (SD 1.02)	Mean = 2.61 (SD 1.0)	NS
Infant gender	9 males, 9 females	11 males, 7 females	NS
HLA I antibodies (AU)	Median = 25.3 (IQR 13–133)	Median = 20.9 (IQR 18–83)	NS
HLA II antibodies (AU)	Median = 4.5 (IQR 10–157)	Median = 7.5 (IQR 3.5–72)	NS
IgE (IU/L)	Median = 35 (IQR 10.5–66.5)	Median = 119 (IQR 44.2–404)	$P = 0.002$
Infants diagnosed with allergic disease by 6 years of age	4/13 (31%)	10/17 (59%)	$P = 0.07$, NS

The characteristics of the population are shown. Values for normally distributed data are shown as mean and standard deviation (SD), and nonparametric data are displayed as median and interquartile ranges (IQR).

HLA, human lymphocyte antigen; NS, not significant.

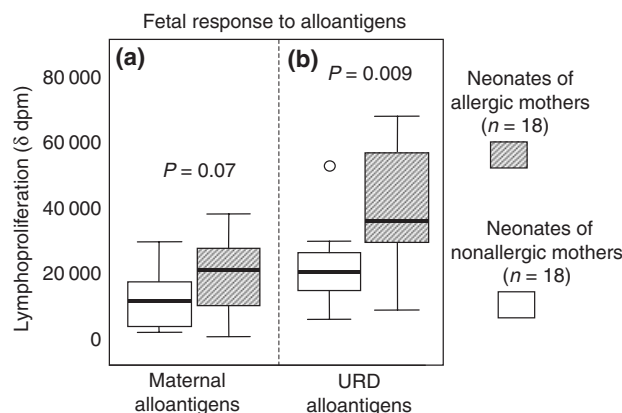


Fig. 1. Comparison of responses to alloantigens by neonates of allergic and non-allergic women. The responses to (a) maternal alloantigens, and (b) unrelated donors is shown for neonates of allergic mothers (shaded bars) or non-allergic mothers (clear bars). Data are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values.

($P = 0.002$) and a trend for lower IFN- γ and higher IL-13 responses to allergen (HDM), but this was not statistically significant (data not shown). These mothers also showed stronger lymphoproliferation to fetal antigens ($P = 0.07$). There were no apparent relationships between maternal allergy and cytokine responses to fetal alloantigens. In contrast, neonates born to allergic mothers showed stronger lymphoproliferative responses to maternal alloantigens ($P = 0.07$) and URD ($P = 0.009$) (Fig. 1). Of note, responses to maternal antigens were significantly lower than for URD ($P < 0.001$).

Associations with the development of allergic disease at 6 years of age

Although infants of allergic mothers were twice as likely to develop allergic disease at 6 years (10/17, 59%) compared with infants of allergic mothers (4/13, 30%), this did not reach statistical significance ($P = 0.07$) probably because of small group numbers. Rather, the development of allergic disease at 6 years was significantly associated with stronger maternal responses to fetal alloantigens. Specifically, maternal lymphoproliferation ($P = 0.008$), IL-13 ($P = 0.02$) and IFN- γ ($P = 0.015$) responses were higher in response to fetal alloantigens if children subsequently developed allergic disease (Fig. 2). Thus, allergic outcomes appeared to be more strongly associated with direct maternal-fetal immune interactions than 'genetic risk' *per se* (as conferred by a history of maternal allergy).

Factors affecting maternal immune responses to fetal alloantigens

We examined the effects of factors that are most likely to influence maternal immune responses to fetal antigens, including previous pregnancies and HLA incompatibility.

Previous pregnancies Maternal responses to fetal alloantigens increased with the number of previous pregnancies. Specifically, the more previous pregnancies, the stronger the proliferation (Kendall $\tau_b = 0.3$, $P = 0.04$), IL-13 ($\tau = 0.26$, $P = 0.07$) and IFN- γ ($\tau = 0.3$, $P = 0.04$) responses to fetal

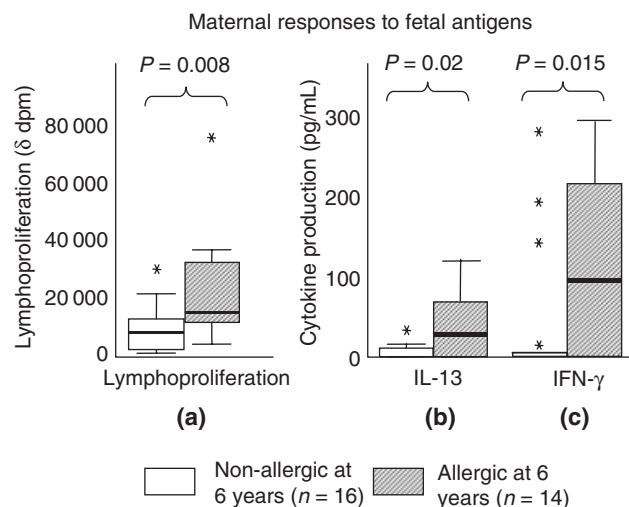


Fig. 2. Comparison of maternal responses to fetal alloantigens according to allergic outcomes at 6 years of age. The maternal (a) lymphoproliferation, (b) IL-13 and (c) IFN- γ responses are compared for pairs where infants were allergic (shaded bars) or non-allergic (clear bars) at 6 years of age. Data are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values.

alloantigens. There were no differences in the number of previous pregnancies in allergic and non-allergic mothers (Table 1).

Maternal human lymphocyte antigen antibodies Because we did not have the resources to HLA type all the mothers and their babies, we measured maternal antibodies to HLA class I and II antigens as an alternative measure of HLA incompatibility. In this population Class I and II antibody levels were not related to the number of previous pregnancies. There was no relationship between the levels of HLA class I antibodies and the immunological responses measured or with maternal or infant allergy (making it unlikely that this was a confounding factor). In this population, there were also no relationships between maternal HLA class II antibody levels and maternal immune responses to the various stimuli (including fetal HLA alloantigens). There was no relationship between HLA class II antibodies and either maternal or infant allergy (data not shown). There were also no differences in the levels of HLA class I or II antibodies in allergic and non-allergic mothers (Table 1).

Infant gender There were 20 male infants and 16 female infants in the study. The only parameter that was affected by gender was the capacity for fetal polyclonal IFN- γ responses (to PHA), which were significantly higher in the males ($P = 0.03$). Because of this, we performed separate analyses of materno-fetal immune relationships in males and females, and showed the same relationships as for the combined population (described below). Gender did not appear to influence any other parameters.

Associations between maternal and neonatal immune responses

Finally, we examined how maternal immune responses were related to neonatal immune responses.

Relationship between maternal human lymphocyte antigen antibodies and fetal immune responses Although there were no relationships between HLA class I antibodies and fetal immune responses (to any stimuli), higher levels of maternal HLA class II antibody were associated with stronger fetal IFN- γ responses to maternal HLA alloantigens ($\tau = 0.3$, $P = 0.03$) and mitogen ($\tau = 0.3$, $P = 0.05$), suggesting that HLA mismatch may be a factor influencing fetal Th1 function. Stronger neonatal IFN- γ responses to PHA mitogen were also associated with stronger neonatal responses to HDM allergen ($\tau = 0.45$, $P = 0.002$).

Relationship between maternal and fetal cellular immune responses The pattern of maternal immune responses to fetal alloantigens was related to the pattern of fetal cytokine production by PHA-stimulated MNC (Table 2). Neonates showed increased capacity to produce IL-13 (in response to polyclonal PHA stimulation) in association with higher maternal Th2 IL-13 responses to fetal alloantigens ($\tau = 0.4$, $P = 0.001$). Fetal polyclonal IL-10 responses were also correlated with maternal IL-10 responses to fetal alloantigens ($\tau = 0.3$, $P = 0.029$). No relationship was seen for IL-5 responses, which were generally low level (i.e. close to or below the detection limit of the ELISA). There were also significant correlations between the maternal and fetal alloantigen lymphoproliferative responses ($\tau = 0.4$, $P < 0.0001$) and IFN- γ responses ($\tau = 0.5$, $P = 0.001$) (i.e. maternal responses to fetal antigens correlated with fetal responses to maternal antigens) (data not shown). As indicated above, HLA

incompatibility appeared to be related to fetal but not maternal Th1 responses.

The study population was not large enough to perform subgroup analyses to separately examine all these relationships in women according to their number of previous pregnancies, maternal allergy or infant gender; however all relationships remained the same when we controlled for possible effects of these factors (maternal allergy, gravidity and infant gender) using partial correlation analyses. Larger studies are needed to examine possible changes in materno-fetal relationships with successive pregnancies.

Relationships between immune response to different stimuli within the mother and the fetus We also examined relationships between responses to different stimuli, as well as the relationships between different cytokines, as shown in Table 3a and b. We were particularly interested in whether fetal responses to maternal alloantigens were related to fetal capacity for cytokine responses. We found that fetal IFN- γ responses to maternal antigens were associated with higher fetal polyclonal IFN- γ responses ($\tau = 0.3$, $P = 0.03$). Fetal IL-13 responses to maternal alloantigens were also correlated with fetal IL-13 responses to allergen ($\tau = 0.4$, $P = 0.004$). Other relationships, also shown in Table 3a and b, also include positive correlations between fetal responses to allergens and mitogen (for both IL-13 and IFN- γ), which were not seen in the adults.

There were strong positive relationships between the level of maternal Th2 IL-13 and Th1 IFN- γ responses to fetal

Table 2. Relationship between maternal and fetal immune response

Maternal responses	Fetal responses								
	Fetal polyclonal responses			Fetal responses to maternal antigens			Fetal allergen responses		
	IL-13	IFN- γ	IL-10	IL-13	IFN- γ	IL-10	IL-13	IFN- γ	IL-10
Maternal responses to fetal antigens									
IL-13	$\tau = 0.42$ $P = 0.001$	$\tau = -0.1$ $P = 0.4$	$\tau = 0.01$ $P = 0.9$	$\tau = 0.1$ $P = 0.4$	$\tau = 0.1$ $P = 0.3$	$\tau = 0.1$ $P = 0.3$	$\tau = -0.1$ $P = 0.5$	$\tau = 0.3$ $P = 0.06$	$\tau = 0.1$ $P = 0.3$
IFN- γ	$\tau = 0.16$ $P = 0.2$	$\tau = 0.2$ $P = 0.13$	$\tau = -0.5$ $P = 0.7$	$\tau = 0.3$ $P = 0.02$	$\tau = 0.3$ $P = 0.02$	$\tau = 0.1$ $P = 0.4$	$\tau = 0.1$ $P = 0.4$	$\tau = -0.2$ $P = 0.3$	$\tau = 0.1$ $P = 0.6$
IL-10	$\tau = 0.02$ $P = 0.8$	$\tau = -0.05$ $P = 0.7$	$\tau = 0.3$ $P = 0.029$	$\tau = -0.1$ $P = 0.3$	$\tau = -0.1$ $P = 0.7$	$\tau = -0.1$ $P = 0.33$	$\tau = -0.2$ $P = 0.8$	$\tau = -0.0$ $P = 0.8$	$\tau = -0.2$ $P = 0.2$
Maternal polyclonal responses									
IL-13	$\tau = 0.0$ $P = 0.9$	$\tau = -0.1$ $P = 0.2$	$\tau = 0.1$ $P = 0.5$	$\tau = 0.2$ $P = 0.1$	$\tau = 0.2$ $P = 0.2$	$\tau = -0.1$ $P = 0.6$	$\tau = 0.1$ $P = 0.8$	$\tau = 0.1$ $P = 0.8$	$\tau = 0.2$ $P = 0.07$
IFN- γ	$\tau = 0.3$ $P = 0.014$	$\tau = 0.5$ $P < 0.001$	$\tau = 0.04$ $P = 0.7$	$\tau = 0.1$ $P = 0.2$	$\tau = 0.2$ $P = 0.1$	$\tau = -0.3$ $P = 0.09$	$\tau = 0.1$ $P = 0.2$	$\tau = 0.2$ $P = 0.2$	$\tau = 0.2$ $P = 0.06$
IL-10	$\tau = 0.3$ $P = 0.016$	$\tau = 0.3$ $P = 0.01$	$\tau = 0.25$ $P = 0.06$	$\tau = -0.2$ $P = 0.1$	$\tau = -0.3$ $P = 0.018$	$\tau = 0.1$ $P = 0.6$	$\tau = -0.1$ $P = 0.3$	$\tau = -0.45$ $P = 0.02$	$\tau = 0.2$ $P = 0.1$
Maternal allergen responses									
IL-13	$\tau = 0.1$ $P = 0.7$	$\tau = -0.4$ $P = 0.02$	$\tau = 0.1$ $P = 0.7$	$\tau = 0.1$ $P = 0.7$	$\tau = -0.2$ $P = 0.2$	$\tau = 0.1$ $P = 0.7$	$\tau = 0.0$ $P = 0.9$	$\tau = -0.5$ $P = 0.04$	$\tau = 0.0$ $P = 0.9$
IFN- γ	$\tau = -0.6$ $P = 0.7$	$\tau = 0.0$ $P = 0.8$	$\tau = -0.1$ $P = 0.3$	$\tau = 0.1$ $P = 0.6$	$\tau = 0.2$ $P = 0.3$	$\tau = 0.2$ $P = 0.2$	$\tau = 0.2$ $P = 0.3$	$\tau = 0.0$ $P = 0.9$	$\tau = 0.2$ $P = 0.3$
IL-10	$\tau = 0.3$ $P = 0.04$	$\tau = 0.2$ $P = 0.3$	$\tau = 0.0$ $P = 0.8$	$\tau = 0.1$ $P = 0.6$	$\tau = 0.0$ $P = 0.8$	$\tau = 0.1$ $P = 0.6$	$\tau = 0.2$ $P = 0.3$	$\tau = -0.2$ $P = 0.4$	$\tau = -0.1$ $P = 0.5$

The Kendall's τ *b* and significance values for relationships between the various maternal responses to various stimuli (fetal alloantigens, mitogens and allergens) and fetal responses (to maternal alloantigens, mitogens and allergens) are shown for each cytokine: IL-13, IFN- γ and IL-10 ($n = 36$). Relationships that are statistically significant (or closely approach statistical significance) are denoted in bold type. IL-5 responses are not shown as these were generally below detection, and no significant relationships were observed.

alloantigens ($\tau = 0.6$, $P < 0.001$), indicating a mixed Th0 response to alloantigens, rather than a clearly polarized Th1 or Th2 response. The same relationship was seen for fetal responses to maternal antigens ($\tau = 0.5$, $P < 0.001$). This mixed pattern of responses was also observed for fetal allergen responses (with positive correlations between Th1 and Th2 cytokines).

Discussion

To our knowledge, this is the first study to describe how variations in maternal cellular immune response to the fetus are associated with fetal immune responses and the subsequent development of allergic disease. This provides support for the recent intriguing observations that cord serum IgE antibody levels are reduced in higher-order offspring [8]. Together, these observations highlight a likely role of variations in materno-fetal interaction in early immune development.

Our findings indicate that fetal Th1 (IFN- γ) function is directly related to HLA mismatch, and the capacity of the fetus to produce IL-13 and IL-10 is directly related to the level of these cytokines produced by the mother in response to fetal alloantigens. This implies that the level and pattern of immunological reactivity at the feto-maternal interface may influence the level and pattern of evolving fetal immune responses. While genetic factors also have a strong influence on fetal immune responsiveness, our findings suggest that the

placental micro-environment could be an equally (if not more) important determinant of immune reactivity in the early postnatal period. This is consistent with speculation that direct maternal influences during gestation may explain the observation that the mother has a stronger influence than the father on the development of allergic disease in the offspring (discussed in [9]).

Although maternal atopy has been associated with variations in fertility, reports have been conflicting [10, 11] and differences in HLA reactivity of atopic mothers with a pre-existing Type 2 (Th2) bias (to specific allergens) have not been investigated previously. Responsiveness to paternal HLA antigens is a key factor controlling the activity of the maternal immune system in pregnancy. Reactivity to paternal/fetal antigens varies widely within the normal population, without overtly compromising the fetus [12]. HLA mismatching between maternal and paternal (fetal) antigens may be a source of immune stimulation during pregnancy, and alter the cytokine balance in the placenta [13]. While profound cytokine shifts threaten pregnancy, the effects of less marked normal variation in cytokine production on fetal immune development need to be explored further. We speculated that mild reactivity between maternal and paternal (fetal) antigens may activate APC to provide an important stimulus for fetal immune maturation (particularly Th1 responses). While our findings confirmed that neonatal Th1 responses are significantly correlated to HLA incompatibility, this did not appear to protect from allergic disease at 6 years, which was also associated with increased alloreactivity. Further studies are needed to explore the reason for this, and how this relates to the observations by many groups that reduced Th1 responses

Table 3. Relationship between the various cytokine responses to different stimuli for (a) the mother and (b) the fetus

	Maternal responses to fetal alloantigens			Maternal polyclonal (PHA) responses			Maternal allergen responses		
	IL-13	IFN- γ	IL-10	IL-13	IFN- γ	IL-10	IL-13	IFN- γ	IL-10
(a) Maternal responses									
Maternal responses to fetal alloantigens									
IL-13		$\tau = 0.62$ $P < 0.001$	$\tau = -0.1$ $P = 0.4$	$\tau = 0.3$ $P = 0.01$	$\tau = 0.1$ $P = 0.6$	$\tau = -0.1$ $P = 0.7$	$\tau = 0.1$ $P = 0.5$	$\tau = 0.0$ $P = 0.9$	$\tau = 0.3$ $P = 0.1$
IFN- γ	$\tau = 0.62$ $P < 0.001$		$\tau = -0.3$ $P = 0.06$	$\tau = 0.34$ $P = 0.01$	$\tau = -0.2$ $P = 0.1$	$\tau = -0.2$ $P = 0.1$	$\tau = 0.1$ $P = 0.4$	$\tau = 0.1$ $P = 0.5$	$\tau = -0.1$ $P = 0.7$
IL-10	$\tau = -0.1$ $P = 0.4$	$\tau = -0.3$ $P = 0.06$		$\tau = 0.0$ $P = 1.0$	$\tau = -0.1$ $P = 0.7$	$\tau = 0.42$ $P = 0.002$	$\tau = -0.4$ $P = 0.04$	$\tau = 0.2$ $P = 0.4$	$\tau = 0.0$ $P = 0.8$
Maternal polyclonal (PHA) responses									
IL-13	$\tau = 0.3$ $P = 0.01$	$\tau = 0.34$ $P = 0.01$	$\tau = 0.0$ $P = 1.0$		$\tau = -0.2$ $P = 0.1$	$\tau = -0.1$ $P = 0.4$	$\tau = 0.1$ $P = 0.5$	$\tau = 0.2$ $P = 0.2$	$\tau = 0.0$ $P = 1.0$
IFN- γ	$\tau = 0.1$ $P = 0.6$	$\tau = -0.2$ $P = 0.1$	$\tau = -0.1$ $P = 0.7$	$\tau = -0.2$ $P = 0.1$		$\tau = -0.2$ $P = 0.1$	$\tau = -0.2$ $P = 0.4$	$\tau = -0.1$ $P = 0.6$	$\tau = 0.3$ $P = 0.2$
IL-10	$\tau = -0.1$ $P = 0.7$	$\tau = -0.2$ $P = 0.1$	$\tau = 0.42$ $P = 0.002$	$\tau = -0.1$ $P = 0.4$	$\tau = -0.2$ $P = 0.1$		$\tau = -0.1$ $P = 0.6$	$\tau = -0.1$ $P = 0.9$	$\tau = -0.1$ $P = 0.7$
Maternal allergen responses									
IL-13	$\tau = 0.1$ $P = 0.5$	$\tau = 0.1$ $P = 0.4$	$\tau = -0.4$ $P = 0.04$	$\tau = 0.1$ $P = 0.5$	$\tau = -0.2$ $P = 0.4$	$\tau = -0.1$ $P = 0.6$		$\tau = 0.1$ $P = 0.7$	$\tau = 0.2$ $P = 0.3$
IFN- γ	$\tau = 0.0$ $P = 0.9$	$\tau = 0.1$ $P = 0.5$	$\tau = 0.2$ $P = 0.4$	$\tau = 0.2$ $P = 0.4$	$\tau = -0.1$ $P = 0.6$	$\tau = -0.1$ $P = 0.8$	$\tau = 0.1$ $P = 0.7$		$\tau = 0.0$ $P = 0.8$
IL-10	$\tau = 0.3$ $P = 0.1$	$\tau = -0.1$ $P = 0.7$	$\tau = 0.0$ $P = 0.8$	$\tau = 0.0$ $P = 1.0$	$\tau = 0.3$ $P = 0.2$	$\tau = -0.1$ $P = 0.7$	$\tau = 0.2$ $P = 0.3$	$\tau = 0.0$ $P = 0.8$	

Table 3. (Continued)

	Fetal responses to maternal alloantigens			Fetal polyclonal (PHA) responses			Fetal allergen responses		
	IL-13	IFN- γ	IL-10	IL-13	IFN- γ	IL-10	IL-13	IFN- γ	IL-10
(b) Fetal responses									
Fetal responses to maternal alloantigens									
IL-13		$\tau = 0.5$ $P < 0.001$	$\tau = -0.1$ $P = 0.8$	$\tau = 0.1$ $P = 0.3$	$\tau = 0.1$ $P = 0.6$	$\tau = -0.1$ $P = 0.6$	$\tau = 0.4$ $P = 0.004$	$\tau = 0.1$ $P = 0.8$	$\tau = 0.24$ $P = 0.05$
IFN- γ	$\tau = 0.5$ $P < 0.001$		$\tau = 0.1$ $P = 0.6$	$\tau = 0.0$ $P = 0.8$	$\tau = 0.3$ $P = 0.03$	$\tau = -0.1$ $P = 0.3$	$\tau = 0.4$ $P = 0.006$	$\tau = 0.3$ $P = 0.2$	$\tau = 0.2$ $P = 0.09$
IL-10	$\tau = -0.1$ $P = 0.8$	$\tau = 0.1$ $P = 0.6$		$\tau = -0.2$ $P = 0.08$	$\tau = -0.1$ $P = 0.3$	$\tau = 0.2$ $P = 0.09$	$\tau = 0.0$ $P = 0.8$	$\tau = -0.2$ $P = 0.02$	$\tau = -0.1$ $P = 0.5$
Fetal polyclonal (PHA) responses									
IL-13	$\tau = 0.1$ $P = 0.3$	$\tau = 0.0$ $P = 0.8$	$\tau = -0.2$ $P = 0.08$		$\tau = 0.2$ $P = 0.03$	$\tau = 0.2$ $P = 0.1$	$\tau = 0.2$ $P = 0.07$	$\tau = -0.2$ $P = 0.1$	$\tau = 0.2$ $P = 0.07$
IFN- γ	$\tau = 0.1$ $P = 0.6$	$\tau = 0.3$ $P = 0.03$	$\tau = -0.1$ $P = 0.3$	$\tau = 0.2$ $P = 0.03$		$\tau = -0.1$ $P = 0.4$	$\tau = -0.3$ $P = 0.01$	$\tau = 0.45$ $P = 0.002$	$\tau = 0.2$ $P = 0.06$
IL-10	$\tau = -0.1$ $P = 0.6$	$\tau = -0.1$ $P = 0.3$	$\tau = 0.2$ $P = 0.09$	$\tau = 0.2$ $P = 0.1$	$\tau = -0.1$ $P = 0.4$		$\tau = -0.2$ $P = 0.04$	$\tau = -0.1$ $P = 0.3$	$\tau = -0.2$ $P = 0.04$
Fetal allergen responses									
IL-13	$\tau = 0.4$ $P = 0.004$	$\tau = 0.4$ $P = 0.006$	$\tau = 0.0$ $P = 0.8$	$\tau = 0.2$ $P = 0.07$	$\tau = -0.3$ $P = 0.01$	$\tau = -0.2$ $P = 0.04$		$\tau = 0.3$ $P = 0.04$	$\tau = 0.2$ $P = 0.02$
IFN- γ	$\tau = 0.1$ $P = 0.8$	$\tau = 0.3$ $P = 0.2$	$\tau = -0.2$ $P = 0.02$	$\tau = -0.2$ $P = 0.1$	$\tau = 0.45$ $P = 0.002$	$\tau = -0.1$ $P = 0.3$	$\tau = 0.3$ $P = 0.04$		$\tau = 0.3$ $P = 0.02$
IL-10	$\tau = 0.24$ $P = 0.05$	$\tau = 0.2$ $P = 0.09$	$\tau = -0.1$ $P = 0.5$	$\tau = 0.2$ $P = 0.07$	$\tau = 0.2$ $P = 0.06$	$\tau = -0.2$ $P = 0.04$	$\tau = 0.2$ $P = 0.02$	$\tau = 0.3$ $P = 0.02$	

The Kendall's τ *b* and significance values which denote the relationships between different aspects of (a) maternal reactivity and (b) fetal reactivity. IL-13, IFN- γ and IL-10 responses are shown ($n = 36$). Relationships that are statistically significant (or closely approach statistical significance) are denoted in bold type. IL-5 responses are not shown as these were generally below detection, and no significant relationships were observed.

at birth are associated with subsequent atopy or atopic risk ([14, 15] and others).

We also postulated that an established predisposition for altered immune responses (i.e. a propensity for Th2 responses in atopic women) may further polarize the cytokine patterns generated towards paternal (fetal) antigens. However, allergic women did not show clear differences in reactivity to paternal antigens, although there was a trend for stronger lymphoproliferative responses. This may have been because of limitations secondary to the sample size. Of note, there was also no polarization of responses. Rather, we observed significant parallel relationships between maternal Th1 and Th2 responses to alloantigens. This argues against a simple dichotomy of immune responsiveness. It remains of significance that the levels of maternal reactivity related directly to fetal responses (even if this was not polarized), supporting our hypothesis that the level of maternal reactivity to the fetus may influence immunological activity of the fetal immune system.

The nature of the observed relationship between maternal responses to fetal antigens and the subsequent risk of allergic disease is unclear. Although we may speculate that more vigorous maternal immune responses in pregnancy may lead to fetal immune activation and increase the risk of subsequent excessive inappropriate immune responses to allergens, there is no direct evidence to support this. It is of note that presymptomatic neonates have stronger lymphoproliferative responses ([16] and others), and that subsequent child-

hood atopy is associated with increased mixed (parallel) Th1 and Th2 responses rather than polarized Th2 responses to allergens ([7, 17] and others). Our new observations should be investigated further in larger long-term studies that examine these relationships at different time points.

The level of immunological reactivity at the materno-fetal interface is also clearly a function of differences in materno-fetal HLA incompatibility, which could account for some of the variations in immune responses in our study. However, we did not observe any relationship between maternal allergy, maternal HLA antibodies and maternal immune responses to fetal (or other antigens). We used maternal HLA antibodies (at term) as a measure of HLA mismatch (because we did not have the resources to HLA type all the individuals in this study). We did observe that HLA Class II antibodies (but not Class I) were positively associated with stronger Th1 responses in the fetus, suggesting that HLA mismatch may influence fetal immune function. The tendency for neonates of allergic mothers to have stronger lymphoproliferation to alloantigens could reflect the previously observed tendency for infants at high risk of atopy to have stronger lymphoproliferative responses to other antigenic stimuli [16]. The significance is unclear, and there is ongoing debate about whether antigen-specific lymphoproliferation in the neonate reflects genuine classical memory responses [18]. In this situation however, the mixed lymphocyte allo-responses we have elicited classically involves naïve T cells.

There appears to be variability in the capacity of women to develop tolerance to paternal antigens with successive pregnancies. Pregnancy may in turn modify maternal immune responses, as recently noted by Harris et al. [19] who observed that successive pregnancies appeared to reduce maternal allergy (symptoms and skin prick reactivity). Our findings also show stronger maternal responses to fetal antigens in women who had more previous pregnancies. It has been observed that, at least in some situations, successive pregnancies have 'more successful Th2 skewing' [20] and lower incidence of Th1-mediated complications [21].

However, in other situations, where Th1 responses are adaptive (i.e. in the protection from placental malaria), higher Th1 (IFN- γ) responses are seen with successive pregnancies and these protect the fetus [22]. Thus, it appears that while all pregnancies have to cope with a degree of maternal/fetal incompatibility, immune responses in pregnancy vary as a result of a complex interplay between maternal immune programming and adaptation to environmental factors. The significance of our findings in this context is unclear and also needs to be investigated further.

This exploratory study focuses on the basic underlying mechanisms of early 'immune programming', and does not attempt to explain the increasing rates of allergic disease, which are clearly because of environmental changes. However, defining maternal/fetal immune interactions in early life is also an essential basis for determining pathways of influence by environmental agents, many of which may exert effects in pregnancy [23]. Our findings provide the first evidence that variations in maternal cellular immune responses during pregnancy are linked to altered fetal immune responses and subsequent allergic propensity. While statistical correlation does not prove cause and effect, our findings emphasize the potential importance of the fetomaternal interface in the pathogenesis of allergic sensitization in early life. These important pilot data highlight the need for more further detailed studies in larger populations. Since the completion of this work, there has been an emerging role for CD4⁺CD25⁺ regulatory T cells in mediated tolerance at the materno-fetal interface [24, 25]. Future studies clearly also need to address the role of these cells in this context.

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