

Modulation of *in vivo* and *in vitro* cytokine production over the course of pregnancy in allergic and non-allergic mothers

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Cytokines secreted during pregnancy may influence immune development of the foetus. This study aimed to determine if maternal allergy alters patterns of systemic cytokine production throughout and after pregnancy. Maternal plasma cytokines and allergen-specific production of interleukin (IL)-10, IL-13 and interferon (IFN)- γ were measured in allergic ($n = 63$) and non-allergic ($n = 70$) pregnant women who had a full set of sequential peripheral blood samples collected at 20-, 30-, 36-wk gestation and 6-wk post-partum. Maternal allergy was strictly defined by both allergen sensitization and doctor-diagnosed asthma, eczema or rhinitis. IL-13 responses to allergen were higher for allergic mothers at all time-points (20 wk: $p < 0.001$; 30 wk: $p = 0.001$; 36 wk: $p < 0.001$; post-partum: $p < 0.001$). For the non-allergic group, IL-13 levels to house dust mite decreased from 20- to 36-wk gestation (Friedman ANOVA $p = 0.012$) and were significantly lower at 36 wk compared with post-partum ($p = 0.002$). In contrast, IL-13 production by allergic mothers did not change from 20 wk through to post-partum. For both allergic and non-allergic mothers, *in vitro* IFN- γ production was lower at all pregnancy time-points compared with post-partum levels. Allergic women had an increased propensity for peripheral blood allergen-specific T helper-2 responses during pregnancy, and failed to downregulate these responses in comparison with non-allergic women. This may be a factor that contributes to the increased risk of atopy in infants born to allergic mothers.

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Cytokines secreted by the placenta and systemically by the maternal immune system are crucially involved in many aspects of successful pregnancy. The hypothesis that modulation towards T helper-2 (Th2)-biased immunity and away from Th1 immunity is characteristic of normal pregnancy (1), has been supported by some studies while other studies have yielded conflicting results (2–7). The gestation-dependent changes in Th1, Th2 and other cytokines levels suggest that this may be an oversimplified view of pregnancy. Th2 cytokines such as interleukin (IL)-4 and IL-13 are upregulated by placental factors which include progesterone (8) and placental growth factor (9).

However, the Th1 cytokine interferon (IFN)- γ may be required during implantation (10). Regulatory cytokines such as IL-10 secreted by trophoblasts are thought to be involved in controlling maternal tolerance to paternal antigens (11). Thus, the complexity and timing of cytokine interactions during pregnancy still requires further investigation.

Currently, the influence of maternal allergy on pregnancy cytokine responses is not fully understood. Allergic diseases are typically characterized by Th2 responses towards environmental allergens. This has led to the speculation that pregnancy may exaggerate the

already Th2-biased allergen-specific responses observed in women with allergic disease (12). To test this hypothesis, we firstly assessed whether the *in vivo* and *in vitro* production of Th1 (IFN- γ), Th2 (IL-13) and regulatory (IL-10) cytokines by peripheral blood mononuclear cells differ between allergic and non-allergic pregnant women. A novel aspect of this study was the use of a strict definition of maternal allergic disease (both positive sensitization and clinical history were required) combined with the longitudinal design from mid-gestation to the post-partum period. Therefore, we also determined if (i) maternal cytokine production modulates from mid- to late-gestation and (ii) whether pregnancy itself has an effect on the level of these cytokines.

Methods

Study design and recruitment criteria

The volunteers in this cohort were derived from a longitudinal study of 169 healthy pregnant women recruited from the private clinics of obstetricians in the Perth metropolitan area, Western Australia between August 2002 and March 2005. At 20-wk gestation, pregnant women were grouped according to allergic status and gravidity and followed up throughout their pregnancy and post-partum. Maternal allergy was defined as a doctor-diagnosed clinical history of asthma, eczema or allergic rhinitis plus a positive skin prick test (SPT) to one or more common allergens. The non-allergic group consisted of mothers who had no history of allergic disease and negative SPT to all tested allergens. Women were considered as 'primigravid' if they had no known previous pregnancies (including miscarriages and terminations). For the purpose of this study, 'multigravid' women were those with one or more previous pregnancies with their current partner (thereby excluding multiparous women having their first pregnancy with a new partner).

Mothers were excluded if: (i) they were aged <18 and >44 yr of age, (ii) there were any maternal or foetal complications (including pre-eclampsia or major congenital anomalies), (iii) parental smoking in pregnancy or during the last 2 yr, (iv) the delivery was pre-term (<36-wk gestation) or (v) they used immunomodulatory medications (including glucocorticoids for threatened pre-term labour). The use of inhalers such as ventolin or topical steroid (e.g. nasal sprays or creams) were recorded, but were not grounds for exclusion.

Collection and cryopreservation of mononuclear cells

Maternal peripheral blood samples were collected from mothers at 20-, 30-, 36-wk gestation and 6-wk post-partum. Thirty millilitres of blood was collected via routine venipuncture of the cubital fossa vein, into an equivolume of heparinized (preservative-free heparin) Roswell Park Memorial Institute (RPMI; Gibco Life Technology, Paisley, UK) tissue culture medium. Mononuclear cells (MNC) and plasma was isolated by Ficoll-Hypaque gradient centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). MNCs were cryopreserved (7.5% DMSO) using established techniques (13) which have been shown not to significantly alter cell function. The plasma fraction was stored at -80°C until required.

Antigen-specific stimulation of MNCs

Vials of cryopreserved MNC were thawed at 37°C then transferred to a 10-ml tube to which cooled RPMI was added drop wise. The cells were pelleted via centrifugation at 500 *g* for 5 min and then resuspended to 1 ml in AIM-V, serum free tissue culture medium (Gibco Life Technology) supplemented with 2-mercaptoethanol (2ME) (4×10^{-5} M final concentration; Sigma, Castle Hill, Australia). Cell viability and concentration was determined by staining with trypan blue (4%).

Cytokine responses to allergens were determined by duplicate culture in 96-well round-bottom plates, cultured at 5×10^5 MNCs/ml in AIM-V supplemented with 2ME alone (control) or with optimal stimulating doses house dust mite (HDM; CSL, Melbourne Australia; 20 $\mu\text{g}/\text{ml}$), and cat hair extract (Alk-Abello, Horsholm, Denmark; 5 $\mu\text{g}/\text{ml}$). For phytohaemagglutinin (PHA; Murex Biotech Ltd, Darford, UK; 1 $\mu\text{g}/\text{ml}$) stimulation, a cell concentration of $2.5 \times 10^5/\text{ml}$ was used. Plates were placed in a 37°C 5% CO_2 incubator for 48 h. Supernatants were collected and stored at -20°C until batch analysis of cytokines.

Cytokine protein detection

Cytokines (IL-10, IL-13, IFN- γ) in cell culture supernatants and plasma were quantified using a 'sandwich-type' time-resolved fluorometry as previously described (14). Matched antibody pairs, (consisting of unlabelled capture antibody and biotinylated detection antibody), and recombinant protein standards (Pharmingen, BD Biosciences, CA, USA) were used at an

optimal concentration established for our laboratory. Data were expressed as concentration in pg/ml. The detection limit of the assay was 5 pg/ml for all cytokines.

Statistical analysis

All statistical analyses were performed using statistical package for social sciences (SPSS; version 13.0 for Mac OS X; SPSS Inc, Chicago, IL, USA). For nonparametric data (i.e. antigen-specific responses) longitudinal changes were first assessed using Friedman ANOVA and group comparisons were analysed by the Mann–Whitney *U*-test. When the Friedman ANOVA was significant ($p < 0.05$) this was followed by pairwise comparisons using Wilcoxon signed ranks tests. When the data could be normalized by log transformation, differences were compared using repeated measures ANOVA followed by paired Student's *t*-tests (for longitudinal data) and independent Student's *t*-tests for group comparisons respectively. Logistic regression was used to assess the effect of potential confounders such as gravidity status, delivery mode and infant gender. Nonparametric data is presented as median and interquartile ranges (IQR). Parametric data are expressed as geometric mean and 95% confidence intervals. A p -value < 0.05 was considered statistically significant for all analyses. Because of the exploratory nature of this study, we did not wish to exclude any important relationships by using stringent correction factors for multiple analyses (such as the Bonferroni correction factor). Therefore, the data has been interpreted conservatively.

Ethics

Ethical approval was granted by Princess Margaret Hospital for Children, King Edward Memorial Hospital, St John of God Hospital and Mercy Hospital ethics committees and all women gave informed written consent.

Results

Characteristics of the cohort

The following analyses were performed on a group of healthy pregnant allergic ($n = 63$) and non-allergic ($n = 70$) mothers who had a full set of sequential blood samples collected at each study time-point (20-, 30- and 36-wk gestation and 6-wk post-partum). All women delivered term singleton infants. The concentration of IL-10, IL-13 and IFN- γ were

also measured in plasma from a subgroup ($n = 112$) of these mothers to assess levels of circulating cytokines. This subgroup consisted of 60 allergic and 60 non-allergic women. These measures of immune status were then examined in relation to (i) maternal allergic status and (ii) pregnancy time-point within each group to assess the effects of these factors on maternal *in vivo* and *in vitro* cytokine production.

The characteristics of these populations are shown in Table 1. There were no significant differences in age at delivery, birth gestation and birth measurements (infant weight, length, head circumference and APGAR score) between allergic and non-allergic mothers. There was a higher proportion of females infants born to allergic mothers (χ^2 $p = 0.053$). Within the allergic group, the predominant condition was allergic rhinitis which affected 47/63 (75%) mothers, while asthma and eczema both affected 23/63 (37%). The number of mothers who had elective caesarean (51/133 or 38% of the combined cohort) was higher than reported for the general population of Western Australia [18.4% in 2004 (15)] which may be caused by a selection bias of volunteers from private obstetricians. Allergic mothers were more likely to require emergency caesarean than non-allergic mothers (χ^2 p -value = 0.033).

Table 1. Characteristics of the cohort

	Non-allergic ($n = 70$)	Allergic ($n = 63$)
Primigravid (%)	29 (41)	28 (44)
Multigravid (%)	41 (59)	35 (56)
Clinical history		
Asthma (%)	–	23 (37)
Allergic rhinitis (%)	–	47 (75)
Eczema (%)	–	23 (37)
Maternal age (yr)	32.9 (s.d. 3.9)	32.3 (s.d. 4.0)
Mode of delivery (%)		
SVD/induced	36 (51)	32 (51)
Elective caesarean	31 (44)	20 (32)
Emergency caesarean	3 (4.3)	11 (16)*
Infant gender		
Male (%)	42 (60)	27 (43)
Female (%)	28 (40)	36 (57)**
Gestational age (wk)	39.0 (s.d. 1.0)	39.5 (s.d. 1.0)
Birth measurements		
Weight (kg)	3.47 (s.d. 0.41)	3.43 (s.d. 0.40)
Length (cm)	50.3 (s.d. 2.1)	49.8 (s.d. 3.0)
Head (cm)	35.1 (s.d. 2.5)	34.6 (s.d. 1.3)
APGAR (5 mins)	9	9
Breast feeding (%)†	60 (86)	55 (87)

Data shown as n (%) or mean (s.d.) for normally distributed data.

SVD, spontaneous vertex delivery. APGAR (median), appearance, grimace, activity and respiration.

* χ^2 p -value = 0.033; ** χ^2 p -value = 0.053.

†Partial or exclusive breast feeding at 6-wk post-partum.

Effect of allergic disease

Maternal MNC cytokine responses to HDM, cat allergen and PHA, as well as plasma cytokines were measured at all time-points in all women of this cohort. As shown in Fig. 1, *in vitro* IL-13 responses to HDM were higher for allergic mothers (i.e. those who had both positive SPT and clinical history) at all time-points (20 wk: $p < 0.001$; 30 wk: $p = 0.001$; 36 wk: $p < 0.001$; post-partum: $p < 0.001$). IL-13 response to cat was only higher in allergic mothers at post-partum (Fig. 2, $p = 0.013$). The observed differences between allergic and non-allergic mothers remained significant when maternal gravidity status, mode of delivery, infant gender, breast feeding at post-partum and maternal age were considered as potential confounders.

In vitro IFN- γ and IL-10 responses did not differ between allergic and non-allergic women at any time-point. There were no significant differences in plasma IL-10, IL-13 or IFN- γ between allergic and non-allergic women.

Cytokine modulation from mid- to late-gestation and the effect of pregnancy

For the non-allergic group, IL-13 levels to HDM showed a significant decrease from 20- to 36-wk gestation (Friedman ANOVA $p = 0.012$, Fig. 1). In contrast, for allergic mothers IL-13 cytokine

production to HDM did not change significantly during these time-points. In additional analyses, only HDM-sensitized mothers ($n = 51$) from the allergic group were compared with all mothers SPT negative to HDM ($n = 81$); however, similar results were obtained. For both allergic and non-allergic mothers, IL-10 and IFN- γ cytokine levels did not change from 20- to 36-wk gestation.

For non-allergic mothers, IL-13 to HDM was significantly lower at 36 wk compared with post-partum ($p = 0.002$, Fig. 1). Plasma IL-13 was also lower during pregnancy (30 and 36 wk) compared with post-partum (Table 2: $p = 0.019$ for 30 wk, $p = 0.035$ for 36 wk) in the non-allergic group. In contrast, for allergic mothers, *in vivo* and *in vitro* IL-13 cytokine levels did not change when comparing pregnancy and post-partum levels. For both allergic and non-allergic mothers, *in vitro* IFN- γ levels were significantly lower during pregnancy compared with post-partum after stimulation with HDM (Fig. 1), cat allergen (Fig. 2) and PHA (Fig. 3). Plasma IFN- γ and IL-10 production did not change significantly between pregnant and post-partum time-points in either group.

Discussion

Allergic disease, which is characterized by a Th2-biased response to allergens, may be a factor that could influence the modulation of Th1/Th2

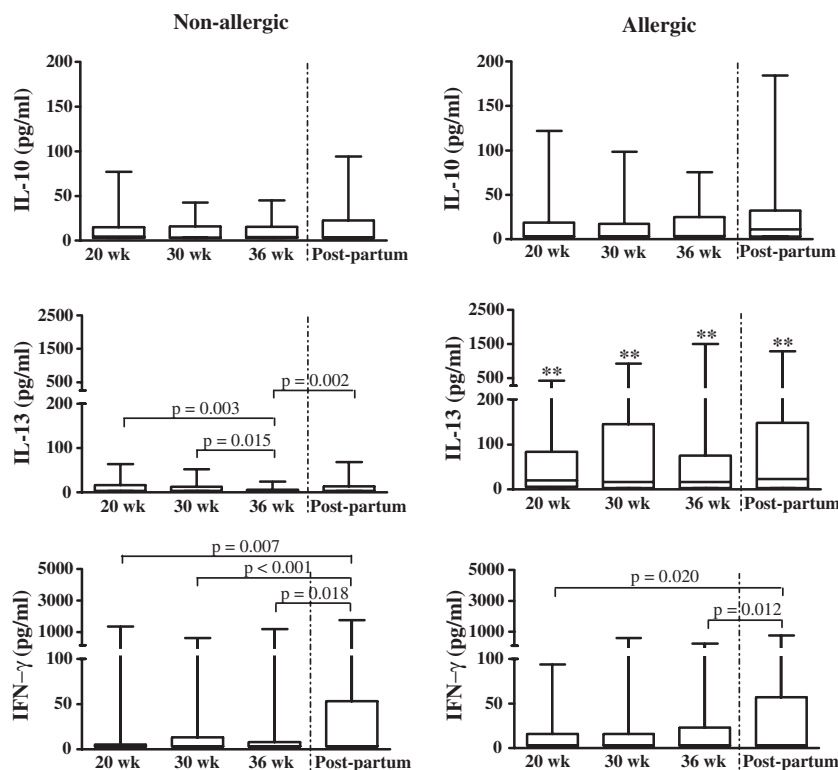


Fig. 1. Changes in house dust mite (HDM) responses during and after pregnancy: peripheral MNC from allergic (SPT pos and clinical history of asthma, rhinitis or eczema) and non-allergic mothers (SPT neg and no clinical history) were collected throughout pregnancy and 6-wk post-partum and stimulated with HDM extract. IL-13, IL-10 and IFN- γ protein concentrations were measured after 48-h incubation and expressed as median, IQR ranges. p-values < 0.05 are shown for differences within groups. $**p < 0.01$ for differences between allergic and non-allergic mothers.

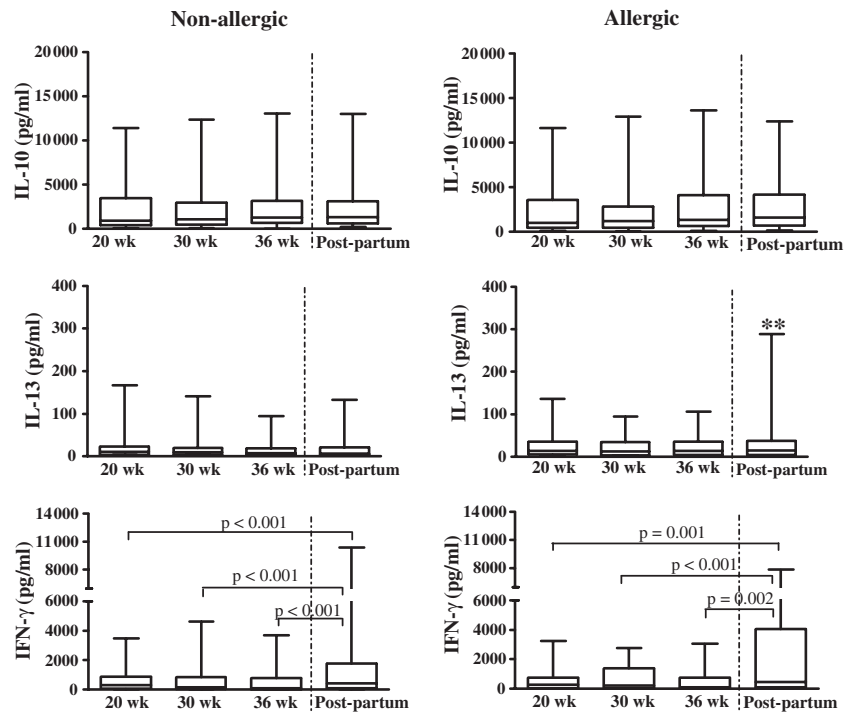


Fig. 2. Changes in cat allergen responses during and after pregnancy: peripheral MNC from allergic (SPT pos and clinical history of asthma, rhinitis or eczema) and non-allergic mothers (SPT neg and no clinical history) were collected throughout pregnancy and 6-wk post-partum and stimulated with cat hair extract. IL-13, IL-10 and IFN- γ protein concentrations were measured after 48-h incubation and expressed as median, IQR ranges. p-values < 0.05 are shown for differences within groups. **p = 0.013 for differences between allergic and non-allergic mothers.

Table 2. Changes in plasma cytokines throughout and after pregnancy

Cytokine	20 wk	30 wk	36 wk	6 wk
(a) Non-allergic group				
IL-10	9.7 (7.3–12.8)	12.5 (8.8–17.8)	11.1 (8.0–15.3)	11.9 (8.9–15.9)
IL-13	34.6 (17.1–69.9)	29.0* (16.0–52.6)	33.0* (18.3–59.6)	41.1 (22.7–74.4)
IFN- γ	20.9 (11.4–38.5)	35.5 (15.4–81.8)	14.5 (8.4–25.2)	25.1 (16.0–39.3)
(b) Allergic group				
IL-10	11.3 (8.3–15.4)	12.4 (8.8–17.3)	14.3 (10.4–19.6)	14.2 (9.8–20.7)
IL-13	33.2 (18.2–60.7)	42.3 (25.8–69.3)	36.7 (22.7–41.1)	42.2 (23.8–74.8)
IFN- γ	37.2 (21.7–63.7)	39.2 (21.1–72.9)	20.3 (11.9–34.6)	43.8 (23.5–81.8)

Data expressed as geometric mean (95% CI) (pg/ml). There were no significant differences between the allergic and non-allergic group.

*Within group difference relative to post-partum levels (p < 0.05).

cytokines during pregnancy. The novel finding of this study was that allergic and non-allergic women showed differential patterns of IL-13 responses to allergen over the course of gestation. Consistent with other reports, allergic women had significantly higher IL-13 Th2 responses to allergens than the non-allergic women (16–19). Importantly, while non-allergic mothers showed downregulation of their (already lower) Th2 responses to HDM from mid- to late-gestation, allergic mothers sustained their high Th2 responses to this allergen throughout and after their pregnancy. This suggests that maternal allergy may alter the regulation of antigen-specific immune responses during pregnancy. In comparison, IFN- γ Th1 responses were not influenced by allergy status and were suppressed

during pregnancy in both groups of women. Factors produced by the placenta such as progesterone (8) and placental growth factor (9), favour Th2 cell development and are thought to contribute to Th1 suppression in the placenta. These factors may also exert immunomodulatory effects on the maternal immune system. We recently noted in the same cohort that foetus-specific IFN- γ responses were reduced in the allergic mothers (20). This suggests that modulation of Th1/Th2 responses during pregnancy may depend on the antigenic stimulus in this population. Therefore, while we conclude that certain allergen-specific Th2 responses are exaggerated in allergic mothers during pregnancy, further study is required to assess Th1 immune modulation in this group of women.

The increased Th2 propensity in allergic women may have downstream effects on foetal immune development. There is now strong evidence that prenatal programming is influenced by maternal allergy and asthma (21, 22). For many years it has been known that maternal asthma can alter foetal growth. Severe asthma is associated with an increased incidence of low-birth-weight babies (23, 24) and even mild asthmatics who did not require steroid treatment had significantly reduced birth-weight percentiles (25). The importance of the *in utero* environment in the pathogenesis of allergic disease is supported by studies showing a greater influence of maternal over paternal atopy on disease risk in

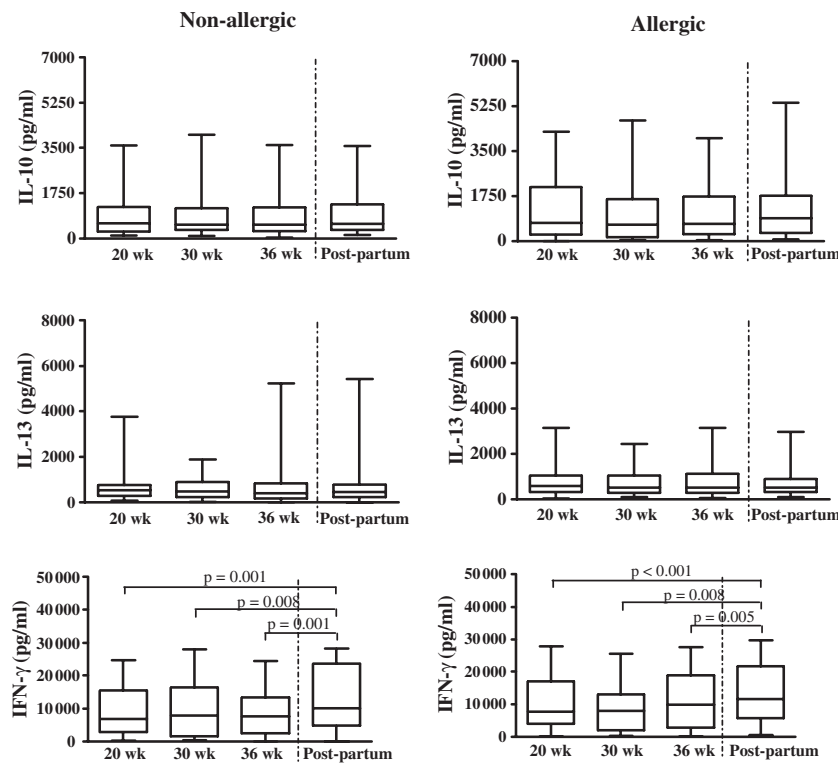


Fig. 3. Changes in PHA responses during and after pregnancy: peripheral MNC from allergic (SPT pos and clinical history of asthma, rhinitis or eczema) and non-allergic mothers (SPT neg and no clinical history) were collected throughout pregnancy and 6-wk post-partum and stimulated with PHA. IL-6, IL-13, IL-10 and IFN- γ protein concentrations were measured after 48-h incubation and expressed as median, IQR ranges. p-values < 0.05 are shown for differences within groups. ** $p < 0.01$ for differences between allergic and non-allergic mothers.

the offspring (26–29). Stimulated cord MNCs from infants of atopic mothers produced higher Th2 or lower Th1 cytokines compared with those from non-atopic mothers (30, 31). Further maternal immune responses during pregnancy closely correlated with cytokine production and IgE at birth (32, 33) and in early childhood (34). There may also be important differences at the placental level in allergic women (30). In the presence of asthma, placental cytokine mRNA expression of IL-10 and proinflammatory cytokines were reduced (35). We speculate that the peripheral responses in women with allergic disease may also alter the cytokine milieu at the maternal–foetal interface, contributing to the altered immune responses of their offspring. However, as there is a paucity of data to support this theory, further studies are required at the placental level in this population.

Overall the range of cytokines produced systemically and in response to antigen in this study supports the need to revise the Th1/Th2 paradigm of pregnancy (36). Our data does not support the notion that pregnancy itself is a ‘Th2 biased’ state, as maternal IL-13 responses were not higher during pregnancy compared with post-partum levels in either group. IFN- γ was readily detected in this study, as have other Th1 cytokines such as IL-2 in previous studies (37). In murine models, an induced state of maternal hyporesponsiveness or tolerance to the allogeneic

foetus has been reported (38). This may occur in humans as regulatory T cells are elevated in the periphery of pregnant women (39). It was expected that production of the regulatory cytokine IL-10 would be increased during pregnancy; however, IL-10 levels did not change between pregnant and post-partum states. While this observation has been confirmed in other studies (40, 41) systemic IL-10 was higher in pregnant women when compared with non-pregnant controls (40). This suggests that IL-10 levels may still remain higher for some time after delivery. Additionally, it is recognized that there are limitations in measuring peripheral immune responses to assess the cytokine milieu of pregnancy. Studies in this laboratory are underway to analyse cytokine modulation at the placental level in allergic women.

In conclusion, allergic women had an increased propensity for allergen-specific Th2 responses during pregnancy, and failed to down-regulate these responses in comparison with non-allergic women. This may be a factor which contributes to the increased risk of atopy in infants born to allergic mothers.

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