

Original article

Effects of maternal allergen-specific IgG in cord blood on early postnatal development of allergen-specific T-cell immunity

Background: A wide body of epidemiologic evidence indicates that as yet unknown maternal factor(s) can influence susceptibility to allergic disease in the offspring. It is also well established that the induction of allergen-specific T-cell memory is frequently initiated *in utero*, and it is likely that maternal factors exert their influence during this period.

Methods: This study examines the relationship between maternally derived allergen-specific IgG subclass antibodies and cellular immune responses (lymphoproliferation and cytokine production) against the same allergens in 49 subjects tested at birth and at 2 years of age. Polyclonal production of the Th1 cytokine IFN- γ was also examined in the cord-blood samples.

Results: At birth, there were positive correlations between both house-dust mite (HDM)- and ovalbumin (OVA)-specific IgG subclass levels in cord blood, maternal atopy, and the magnitude of perinatal lymphoproliferative responses to respective allergens. Inverse relationships were also observed between cord-blood IgG antibody titres and allergen-specific production of some Th2 cytokines. However, there were no consistent relationships between cord-blood allergen-specific IgG antibodies and subsequent immune responses to allergens when the same subjects were retested at 2 years of age. An inverse relationship was observed between maternal history of atopy and perinatal IFN- γ production capacity.

Conclusions: Our results suggest that transplacental transfer of allergen-specific IgG antibody is unlikely to be a major mechanism for maternal regulation of allergen-specific immunity in infancy. An alternative possibility is that maternal effects may operate by influencing IFN- γ production by T cells in the offspring.

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With the increasing incidence and burden of atopic disease, there is growing interest in early life events which may contribute to maturation of allergen-specific immunologic memory. Recent evidence that allergen-specific immunity is initiated *in utero* in most individuals (1) has stimulated further interest in factors which may influence immune development in the gestational environment, particularly maternal factors. This paper addresses the growing speculation that maternal allergen-specific IgG antibodies which cross the placenta may influence the developing infant immune responses to the same allergens.

Levels of maternal allergen-specific IgG reflect both patterns of allergen exposure, and patterns of maternal immune responses. In adults, atopic disease (2) and chronic allergen exposure are associated with higher levels of aeroallergen-specific IgG4, which appears to be promoted by Th-2 (IL-4) cytokines (3–5). In animal models, IgG responses by immunized mothers inhibit

the production of IgE in offspring (6). The evidence in humans is less clear, and there has been no comprehensive analysis of effects on underlying cellular immune responses. Although early studies suggested that high levels of (maternal) food-allergen-specific IgG antibodies in cord sera protect against infant atopy (7–9), subsequent reports have shown positive associations between cord-blood allergen-specific IgG levels and later atopy (10). A more recent study (11) showed that cord-blood IgG subclasses of infants with atopic mothers are correlated with allergen-specific subclass levels at 6 months of age, suggesting that maternal IgG responses may influence developing IgG responses to allergen in the infant. The potential effects of maternal IgG subclass antibodies on cellular immune responses in the offspring that drive the allergic process have not yet been studied in detail. Elucidation of these interrelationships was the principal aim of this study.

An alternative possibility is that other (as yet

unknown) maternally derived factor(s) may influence the development of allergen-specific Th-cell memory via mechanisms which are not allergen-specific; for example, by influencing the functions of foetal antigen-presenting cells, or via polyclonal influences on the overall T-helper (Th)-cell population. In relation to the latter possibility, we and others (reviewed in Ref. 12) have provided evidence that atopic predisposition is associated with decreased capacity for production of the Th1 cytokine IFN- γ during infancy. Accordingly, a secondary aim of this study was to examine the relationship between maternal atopic status and IFN- γ production capacity in the offspring.

Material and methods

Subjects

Sixty healthy pregnant women were recruited at term, just prior to the delivery of their infants. This was an unselected group of consecutive presentations to the King Edward Memorial Hospital Centre for Women's Health in Perth, Western Australia, over a 3-month period. All pregnancies were uncomplicated. The infants were reviewed at 2 years of age, to assess their immunologic development. Forty-nine mother-infant pairs remained in the study at the 2-year follow-up. To determine the relationship between maternal IgG levels in cord blood and postnatal infant cellular immune responses at 2 years, the parameters of all 49 infants were used in correlation studies. This population included atopic ($n=25$) and nonatopic mothers ($n=24$). Maternal atopic status was confirmed by medical history, and measurement of maternal total serum IgE, maternal specific serum IgE (to house-dust mite [HDM], food mix, and grass mix), and maternal skin prick test (SPT) reactivity (13, 14) to extracts of HDM, grass mix, cat, mould, milk, egg, and peanut (all supplied by Hollister Stier, Miles, Inc., Pharmaceutical Division, Elkhart, USA). All maternal testing was done several days after the delivery of the infant. Mothers were considered atopic if they had a medical history of atopy, confirmed by SPT and/or RAST. Additional family history of atopy was also determined by maternal interview. First-degree relatives were considered atopic if they had a doctor-diagnosed allergic disease (SPT were not performed). The limitations of this are recognized. Infants were considered to have atopic heredity (Fig. 2), if they had a first-degree relative with atopy.

A history of cigarette smoking and allergen exposure was also collected by a maternal questionnaire at the postnatal visit, including dietary history (duration of breast-feeding, and age of introduction of various foods). The inaccuracies and limitations of these techniques are recognized, and data were interpreted accordingly. HDM exposure is virtually universal in coastal Australia, and house-dust sampling was beyond the scope of this study.

The main focus of this study was on the association between maternal antibodies and cellular immune responses, rather than on clinical atopic outcome (which is impossible to define at this age). Thus, although clinical information was collected (by history and physical examination) on all infants at follow-up (doctor-diagnosed wheezing, eczema, and urticaria), it is not possible at this age to classify infants reliably as atopic or nonatopic. Therefore, this was not done.

Sample collection

Cellular immune responses were assayed at birth and at 2 years of age by the methods outlined below. Cord blood was collected, as previously described (1), from the placental vessels, and peripheral blood samples were collected from the same infants at 2 years of age. Cord-blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient centrifugation (at 1350 rpm for 30 min). Cell cultures were performed with serum-free AIM V medium (Gibco). Plasma was collected from each sample and frozen for transport to Sweden (at -80°C).

Lymphoproliferation

CBMC were cultured in triplicate for 7 days (PBMC for 6 days) in microwells at 10^6 cells/ml in serum-free medium, as previously detailed (1), either alone or in the presence of HDM extract (10 $\mu\text{g}/\text{ml}$) and ovalbumin (OVA) (100 $\mu\text{g}/\text{ml}$). All culture times and concentrations were identified as optimal for *in vitro* T-cell stimulation in preliminary experiments using CBMC. DNA synthesis was measured as incorporation of ^3H -thymidine at the 7-day time point, after pulse labelling at 6 days. Data were expressed as delta dpm per culture, designating levels of incorporation above background (unstimulated) control cultures. Responses which were more than twice backgrounds and more than 1000 dpm above backgrounds were considered positive (15).

Cytokine measurements

Detailed methods of cytokine analysis after allergen stimulation for 24 h have been reported previously (1, 16). Briefly, CBMC were cultured for 24 h in medium alone, or supplemented with optimal stimulating concentrations of HDM allergen (10 $\mu\text{g}/\text{ml}$), OVA (100 $\mu\text{g}/\text{ml}$), or PHA (1 $\mu\text{g}/\text{ml}$). Supernatants were assayed for the presence of IL-6, IL-10, IL-13, and IFN- γ with commercial ELISA kits (CLB Laboratories, Amsterdam, The Netherlands). IL-4, IL-5, IL-9, and IFN- γ , together with β -actin, were also measured as mRNA in cell pellets by standardized semiquantitative RT-PCR, as previously described (1). RNA was extracted from cell pellets with RNeasy B, and cDNA

was transcribed with 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 , β -actin plus Tthplus DNA polymerase, and were performed in a programmable thermocycler (Perkin-Elmer, California, USA). Individual cycle analyses were performed at each age with each primer set, and cycle numbers chosen that were in the linear phase of the respective amplification reactions. Verification of the expected size of the PCR products was obtained by analysis in 1.5% agarose gels. Samples of the PCR products were manually dot-blotted onto nylon membranes, and hybridized overnight with biotinylated, double-stranded probes, and binding was visualized by chemiluminescence with a commercial kit. Probe binding was quantified by computer-assisted densitometric scanning. The intensity of each test sample was expressed as a ratio relative to that of its respective β -actin control.

Measurement of total and specific IgE levels

Total serum IgE and allergen-specific IgE measurements were performed on infant plasma collected at 2 years of age. These measures were kindly performed by the immunology department at Princess Margaret Hospital under the direction of Dr Richard Loh, with a standardized commercial fluoroimmunoassay (Pharmacia CAP System, Pharmacia, Sweden). HDM-specific IgE to *Dermatophagoides pteronyssinus* was measured. The food-allergen-specific IgE responses measured were to egg white, milk, fish (cod), wheat, peanut, and soya bean. The grasses used were couch, ryegrass, timothy, meadow, Johnson, and Bahia. Total serum IgE was measured with covalently bound anti-IgE to bind IgE in patient serum. The standards used for quantification included a Pharmacia serum IgE control, a pooled human serum internal control (collected locally), and a high IgE internal control.

Measurement of allergen-specific IgG

Measurement of HDM-specific IgG, IgG1, and IgG4 antibodies to OVA and Der p 1 (in plasma from cord blood and 2-year infant samples) was performed as described previously (11). Briefly, Costar 3590 microtitre plates (Life Technologies AB, Täby Sweden) were coated with 25 mg/ml OVA (grade VI, Sigma Aldrich, Stockholm, Sweden) or 2 mg/ml Der p 1 in PBS. Free binding sites were blocked with human serum albumin (Chemicon, Stockholm, Sweden). Plasma or reference serum samples or buffer without added serum (blanks) were added to duplicate wells, and then monoclonal antibodies to the human IgG subclasses (IgG [clone HP6064], IgG1 [clone HP6069], and IgG4 [clone HP6011], Bio Zac AB, Järfälla, Sweden) were added. These clones were all recommended by WHO/IUIS (17). Alkaline-phosphatase-conjugated rabbit anti-

mouse IgG (Sigma-Aldrich, Stockholm, Sweden) was added, and *p*-nitrophenyl phosphate (pNPP) (Sigma Aldrich, Stockholm, Sweden) was used as substrate. The optical density (OD) was read at 405 nm. Values were expressed as arbitrary units (AU) deduced from the ODs of the reference serum curve, and corrected for the dilution factor, after subtracting the blanks. The reference sera, which were calibrated according to a semiquantitative method as described previously (11), were from an individual with known high antibody titres to OVA and from patients undergoing HDM immunotherapy, respectively. The titres in the reference sera were, for antibodies to OVA, IgG1 2789 AU and IgG4 4884 AU, and for antibodies to Der p 1, IgG1 1216 AU and IgG4 624 AU.

Samples in which levels of allergen-specific IgG4 antibodies were below detection by the conventional ELISA were reanalysed with a more sensitive enzyme-amplified ELISA, as previously described (11). Coating, blocking, and addition of human serum samples, monoclonal antibodies to human IgG4, and alkaline phosphatase-conjugated rabbit antimouse IgG were done as described above. For the substrate reaction, AMPAK kits (Dakopatts AB, Älvsjö, Sweden) were used. NADPH in diethanolamine buffer was allowed to react with the phosphatase for 20 min followed by 10-min incubation with amplifier solution. OD was measured at 492 nm. Values were expressed as arbitrary units (AU) deduced from the ODs of the reference serum curve, after subtracting the blanks. Generally, intra-assay coefficients of variation were <5% and interassay coefficients of variation were <15% in the conventional and enzyme-amplified ELISA.

Analysis

Nonparametric tests were used to examine relationships between continuous variables (Spearman correlation coefficients) with SPSS (Statistical Package for the Social Sciences). The Mann-Whitney U-test was used to compare two groups.

Ethical considerations

Cord-blood collection took place at King Edward Memorial Hospital, Subiaco, Western Australia. The infants were followed up at Princess Margaret Hospital for Children, Subiaco, Western Australia. The study was approved by the ethics committees of both centres.

Results

Cord-blood IgG subclass levels and maternal atopy

Neonates with a maternal history of atopy had significantly higher cord-blood HDM-IgG4 levels ($P=0.007$) than infants with no maternal atopy (Fig. 1a). There was also a tendency for neonates with

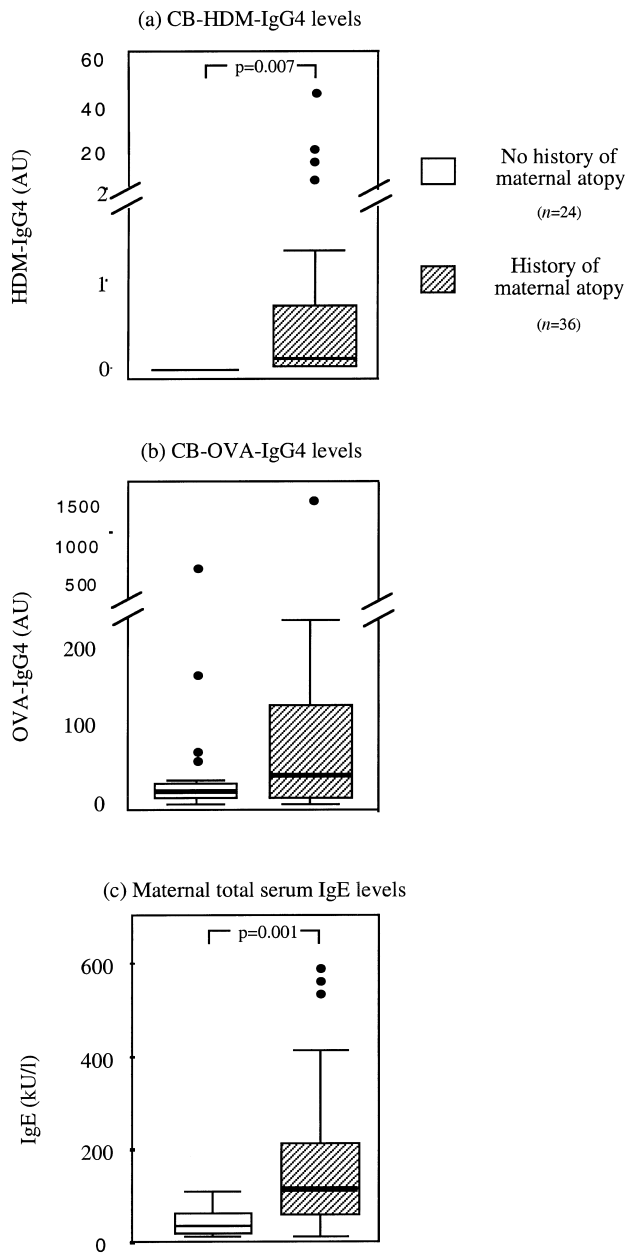


Figure 1. Comparison of total maternal serum IgE, and allergen-specific CB IgG4 levels, by maternal atopic history. Allergen-specific CB-IgG4 against HDM (panel a) and OVA (panel b) in cord blood, and maternal IgE (panel c) were compared for maternal atopic history, as described in text. Data for each group are shown as 10th, 25th, 50th, 75th, and 90th percentiles as well as outliers, and comparisons were made by Mann–Whitney U-test.

maternal atopy to have higher OVA-IgG4 levels (Fig. 1b), than neonates with no maternal atopy ($P=0.08$). Consistent with this, there was a significant positive correlation between maternal IgE levels and both HDM-IgG4 ($r=0.4$, $P=0.003$) and OVA-IgG4 ($r=0.34$, $P=0.024$) in cord blood. A similar relationship was present for maternal HDM-SPT wheal size and HDM-IgG4 ($r=0.64$, $P<0.0001$). HDM-IgG1 levels also tended to be higher with maternal atopy ($P=0.06$).

There were no differences in OVA-IgG1 levels between groups compared by maternal atopy or family history. As anticipated, maternal IgE levels were significantly higher in mothers with atopic history (Fig. 1c) than mothers without atopy ($P=0.001$). Mothers who smoked tended to have higher total serum IgE levels ($P=0.06$), but smoking was not related to cord-blood allergen-specific IgG subclass levels in this population.

The influence of cord-blood IgG levels on perinatal immune responses

Levels of allergen-specific IgG in cord blood correlated with the magnitude of CBMC lymphoproliferative responses by the foetus (Table 1). There were positive correlations for OVA-IgG, OVA-IgG4, and HDM-IgG1 and the magnitude of lymphoproliferative responses to the same allergen by CBMC. A similar trend of correlation was seen between HDM-IgG4 and proliferation to HDM ($P=0.06$). There were negative associations between HDM-IgG1 and IL-4 mRNA responses, HDM-IgG4 and IL-5 mRNA responses, OVA-IgG and IL-5 mRNA responses, and OVA-IgG (and OVA-IgG4) levels and IL-13 responses (all shown in Table 1).

Neonates at high atopic risk (positive family history) had lower polyclonal IFN- γ responses (Fig. 2), but this was statistically significant only for infants with maternal atopy ($P=0.03$). These high-risk infants also had significantly lower IFN- γ responses than other high-risk neonates who had no maternal atopy ($P=0.04$). Thus, in this small population, impaired IFN- γ responses were more a function of maternal atopy than general family heredity (atopic risk) per se.

Associations between cord-blood IgG levels and postnatal immune responses

Cord-blood allergen-specific IgG, IgG1, and IgG4 levels were not related to subsequent patterns of cellular immune response to allergens at 2 years of age. Specifically, cord-blood antibody levels did not show any consistent relationships to allergen-specific cytokine responses (IL-4, IL-6, IL-9, IL-10, IL-13, or IFN- γ) in later infancy. The only exception was a negative relationship between total CB-OVA-IgG levels and OVA-IL-5 responses at 2 years of age ($r=-0.4$, $P=0.02$).

There were no significant associations between cord-blood allergen-specific IgG subclass levels and the magnitude of allergen-specific lymphoproliferative responses at 2 years of age.

Cord-blood IgG subclass levels were positively related to later allergen-specific IgG response. Babies with higher OVA-IgG4 in cord blood were significantly more likely to have higher levels of OVA-IgG4 ($r=0.42$, $P=0.02$) and OVA-IgG1 ($r=0.34$, $P=0.04$) levels at 2 years of age. This positive association between the levels

Table 1. Relationships between allergen-specific IgG levels and cellular immune responses (a) at birth and (b) at 2 years. In each case, IgG, IgG1, or IgG4 levels were compared with lymphoproliferation (delta dpm) and cytokine responses to respective allergens (HDM and OVA) and expressed as Spearman (nonparametric) correlation coefficient (significance values are shown in parentheses). Allergen-stimulated IL-4 mRNA, IL-5 mRNA, IFN- γ mRNA, and IL-13 protein were measured as levels above background unstimulated cultures after 24 h

	Cord-blood IgG levels					
	HDM			OVA		
	IgG	IgG1	IgG4	IgG	IgG1	IgG4
Allergen-specific responses at birth						
Lymphoproliferation	0.2 (NS)	0.3 ($P=0.03$)	0.26 ($P=0.06$)	0.32 ($P=0.02$)	0.1 (NS)	0.42 ($P=0.01$)
IL-4 mRNA	-0.15 (NS)	-0.3 ($P=0.03$)	-0.18 (NS)	-0.3 (NS)	-0.22 (NS)	-0.22 (NS)
IL-5 mRNA	-0.03 (NS)	-0.14 (NS)	-0.4 ($P=0.006$)	-0.32 ($P=0.02$)	-0.2 (NS)	-0.19 (NS)
IL-13	-0.02 (NS)	-0.12 (NS)	-0.18 (NS)	-0.34 ($P=0.01$)	-0.19 (NS)	-0.38 ($P=0.01$)
IFN- γ mRNA	0.1 (NS)	-0.02 (NS)	-0.13 (NS)	-0.04 (NS)	-0.19 (NS)	-0.03 (NS)
Allergen-specific responses at 2 years						
Lymphoproliferation	-0.07 (NS)	-0.08 (NS)	-0.07 (NS)	-0.29 (NS)	-0.42 ($P=0.02$)	-0.3 (NS)
IL-4 mRNA	0.18 (NS)	0.26 (NS)	-0.01 (NS)	-0.03 (NS)	-0.09 (NS)	0.00 (NS)
IL-5 mRNA	0.23 (NS)	0.14 (NS)	-0.08 (NS)	-0.4 ($P=0.02$)	-0.3 (NS)	-0.18 (NS)
IL-13	0.01 (NS)	0.04 (NS)	-0.07 (NS)	0.01 (NS)	-0.09 (NS)	0.10 (NS)
IFN- γ mRNA	-0.02 (NS)	-0.03 (NS)	0.15 (NS)	0.03 (NS)	-0.13 (NS)	0.00 (NS)

of allergen-specific IgG subclass antibody at birth and at 2 years was a consistent observation, but did not always achieve statistical significance.

Although clinical symptoms are nonspecific at this age and cannot be used to define atopic status, there was no association between cord-blood allergen-specific IgG levels and the presence of eczema, wheezing, or urticaria at 2 years of age. At 2 years of age, there were no differences in IgG subclass levels when infants were compared on the basis of maternal atopic status or clinical symptoms. The number of children with detectable allergen-specific IgE (by RAST or SPT) was too small for separate analysis. Although the inaccuracies of dietary records were recognized, the age of introduction of hen's egg into the diet was negatively associated with the IgG4:IgG1 ratio at 2 years ($r = -0.41$, $P=0.02$); i.e., more delayed exposure was associated with lower IgG4:IgG1 ratios. The duration of breast-feeding did not appear to be related to either the cellular or IgG allergen-specific responses.

Discussion

This study provides the first evidence that cellular immune responses to specific allergens in late infancy are not significantly influenced by levels of maternal allergen-specific IgG reaching cord blood.

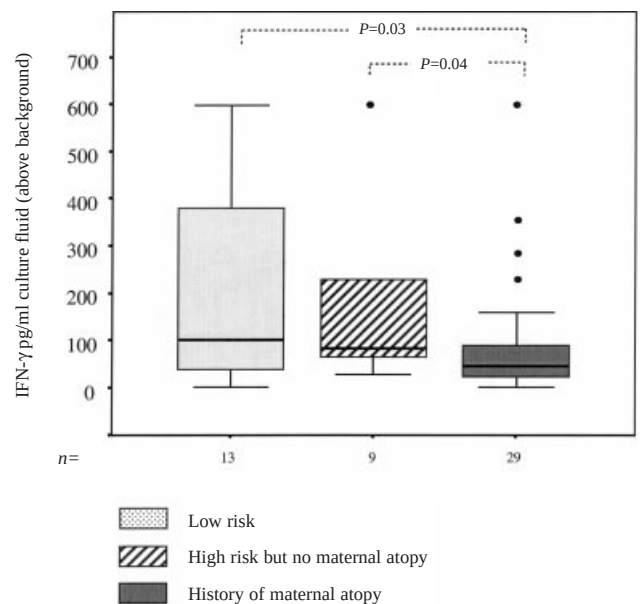


Figure 2. Comparison of perinatal (cord blood) IFN- γ responses to PHA by maternal atopic history and atopic heredity. PHA-stimulated CBMC IFN- γ responses of neonates were compared on basis of maternal atopic history and atopic heredity, as described in text. Data are shown as 10th, 25th, 75th, and 90th percentiles as well as outliers. Comparisons were made with Mann-Whitney U-test (comparing two groups) or Kruskal-Wallis test (comparing three groups).

While cord-blood IgG antibody levels were positively associated with perinatal allergen-specific lymphoproliferative responses, and also displayed a negative relationship with some Th2 cytokine responses, these relationships did not persist into later infancy. It is possible that maternal IgG antibody mediates these early immunologic effects via Fc receptor binding on foetal cells (including B cells, NK cells, and phagocytes). However, these effects appear to be transient, as at 2 years there was no longer any relationship between maternal IgG and cellular immune responses. There is a previously reported association between atopic risk (family atopy) and higher proliferative responses at birth (18). It is unclear whether the association between maternal IgG4 and perinatal lymphoproliferation noted in the present study is a primary (causal) association, or is only secondary to independent associations between both factors (IgG4 and perinatal lymphoproliferation) and maternal atopy.

Although not its primary aim, this study also confirms the previously observed association between allergen-specific IgG4 levels and maternal atopic status (2, 19). This was strongest for aeroallergen (HDM), but was also evident for food allergen (OVA), even in the absence of documented food reactivity (by history, RAST, or SPT). HDM-IgG1 levels in cord blood were also associated (but nonsignificantly) with maternal atopy. The role of maternal exposure to HDM was difficult to determine in this study (although this is

fairly ubiquitous in coastal Australia). The proportionally higher infant OVA-IgG4 levels (at 2 years) in association with earlier introduction of dietary egg may reflect a relationship between levels of environmental allergen and IgG4 levels, but this should be confirmed in studies that more accurately assess dietary intake.

Maternal atopy is a strong risk factor for infant atopy; in a number of studies (reviewed in Ref. 20), it had a stronger influence than paternal atopy. Given that, as demonstrated in this study, transplacental antibody transfer does not appear to contribute significantly to these maternal effects, the question arises as to what alternative immunologic pathways might be involved. Earlier studies from our laboratory have established that a hallmark of the atopic phenotype in infancy is transiently decreased capacity to produce the Th1 cytokine IFN- γ (21). Subsequently, numerous groups have demonstrated impaired IFN- γ responses at birth in HR neonates. The findings in Fig. 2 suggest that these maternal influences are

positively associated with this maturation defect, although this should be confirmed in larger studies. As noted earlier (12), the maturational defect in IFN- γ production capacity in atopic family history-positive neonates appears to be an exaggeration of Th2-skewing (i.e., Th1-suppressive) mechanisms which operate in the foetal environment to protect the integrity of the foetomaternal interface. The underlying mechanism(s) are only partially understood, and are believed to involve contributions from both the placenta and the mother. We speculate that one such mechanism may be hyperactive in atopic mothers, and be responsible for the effects shown in Fig. 2.

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