

Original article

Presymptomatic differences in Toll-like receptor function in infants who have allergy

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Background: Microbial exposure might play a key role in allergy development, but little is known about the role of Toll-like receptors (TLRs).

Objective: This study explored the association between neonatal TLR microbial recognition/function, allergy risk (maternal allergy), and prospective allergy development.

Methods: Cord blood mononuclear cells (n = 111) were cultured either alone or with optimal concentrations of TLR ligands: lipoteichoic acid (TLR2), polyinosinicpolycytidylic acid (TLR3), LPS with IFN- γ (TLR4), flagellin (TLR5), imiquimod R837 (TLR7), or CpG (TLR9). Cytokine responses were assessed in relation to allergy risk (maternal allergy) and allergy outcomes (sensitization, food allergy, and atopic dermatitis) at 12 months of age.

Results: Maternal allergy (n = 59) was associated with significantly higher neonatal IL-12 and IFN- γ responses to TLR2, TLR3, and TLR4 activation, whereas TNF- α and IL-6 responses to TLR2, TLR4, and TLR5 activation were significantly higher in newborns who subsequently had allergic disease (n = 32). Notably, consistent with previous reports, newborns who had disease had lower T_H1 IFN- γ response to mitogens (PHA).

Conclusion: Allergic disease was associated with increased (rather than decreased) perinatal TLR responses. Further studies are needed to determine how these responses track in the postnatal period and whether this relative hyperresponsiveness is a product of intrauterine influences, including maternal atopy, functional genetic polymorphisms, or both. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Toll-like receptors, hygiene hypothesis, cord blood, allergic disease, cytokines, endotoxin

Abbreviations used

APC: Antigen-presenting cell
mDC: Myeloid dendritic cell
MFI: Mean fluorescence intensity
MxA: Myxovirus resistance protein A
NK: Natural killer
pDC: Plasmacytoid dendritic cell
SPT: Skin prick test
TLR: Toll-like receptor
UBC: Ubiquitin-conjugating enzyme E2D2

Reduced early microbial exposure has become a leading candidate explaining the escalating rate of allergic disease in the last 40 years, thus generating intense interest in the interaction between the microbial environment and key microbial recognition pathways, namely Toll-like receptor (TLR) function. Both genetic polymorphisms associated with TLR function¹ and environmental factors, such as reduced microbial exposure,^{2,3} can predispose to allergic disease through effects on innate immune pathways. TLRs are found on many cells involved in immediate host defence, including neutrophils; natural killer (NK) cells; antigen-presenting cells (APCs), which play a critical role in programming adaptive T-cell responses; and CD4⁺CD25⁺ regulatory T cells,⁴ which are important in the prevention of hyperreactive immune responses.⁵ Thus TLR-mediated activation of both APCs and regulatory T cells might play an important role in reducing the risk of T_H2-mediated allergic responses.^{5,6} Together, these findings suggest that alterations in TLR function, either as a result of differences in early environmental exposures or genetic factors, have an effect on the subsequent development of adaptive immune function and allergy.

Maternal allergy, which is a well-recognized risk factor for allergic disease, has been associated with reduced neonatal TLR2 responses⁷ and lower levels of TLR2, TLR4, and CD14 mRNA in cord blood samples.⁸ Neonates at “high risk” of allergy have also been noted to have altered generation of putative regulatory T-cell populations after LPS stimulation, presumably through TLR4 pathways.⁹ To our knowledge, this is the first study to prospectively examine for presymptomatic differences in the early function of a wide range of these critical microbial recognition pathways in children who subsequently have allergic disease.

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METHODS

Study design and recruitment criteria

This was a prospective study of 169 pregnant women from Perth, Western Australia. Allergic mothers were defined based on (1) a clinical history of asthma, eczema, or allergic rhinitis (including a medical diagnosis) and (2) confirmed allergen-specific IgE, as determined by using skin prick tests (SPTs). Women were recruited at 20 weeks of pregnancy, when allergy testing was performed. They had interview questionnaires at 20, 30, and 36 weeks of pregnancy and again at 6 weeks in the postnatal period (for environmental and health data). Perinatal data were collected directly from the hospital charts, and women did not receive questionnaires around childbirth. Only nonsmoking women with no significant pregnancy complications were included. Ethical approval for the study was granted by all participating hospitals.

Assessment of polyclonal mitogen responses

Mononuclear cells from cord blood collections were isolated by means of Ficoll-Hypaque gradient centrifugation and cryopreserved with established techniques.¹⁰ PBMCs (1×10^6 /mL) were cultured alone or with PHA mitogen (HA16; Murex Biotech Ltd, Dartford, United Kingdom) for 48 hours at a final concentration of $1 \mu\text{g/mL}$, as described previously,¹⁰ to assess polyclonal T-cell responses.

Assessment of TLR function

Cord blood mononuclear cells were cultured alone or with optimized doses of lipoteichoic acid ($20 \mu\text{g}$, $1 \mu\text{g/mL}$; InvivoGen, San Diego, Calif) as a TLR2 ligand, poly I:C ($30 \mu\text{g/mL}$; Sigma, Victoria, Australia) as a TLR3 ligand, LPS (10 ng/mL ; *Escherichia coli* LPS, Sigma) as a TLR4 ligand, flagellin ($1 \mu\text{g/mL}$, InvivoGen) as a TLR5 ligand, or imiquimod R837 ($10 \mu\text{g/mL}$, InvivoGen) as a TLR7 ligand. Cord blood mononuclear cells were cultured either alone or together with optimal stimulating doses of CpG ODN (CpG B ODN; $1.66 \mu\text{g/mL}$; Coley Pharmaceutical Group, Ottawa, Ontario, Canada) or non-CpG ODN control ($1.66 \mu\text{g/mL}$, Coley Pharmaceutical Group) to determine TLR9 responses. All cultures were plated in duplicate in 96-well, round-bottom plates (2×10^5 /mL for TLR5, TLR7, and TLR9 and 1×10^6 /mL for all other ligands) in $250 \mu\text{L}$ of RPMI (Gibco Life Technology, Grand Island, NY) plus non-heat-inactivated 10% FCS (Australian Biosearch, Perth, Australia), as previously reported,¹¹ and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours (except CpG ODN cultures, which were incubated for 48 hours). Parallel LPS (TLR4) cultures were performed (1) unprimed (for detecting IFN- γ) and (2) primed with rIFN- γ (10 ng/mL) 3 hours before the addition of LPS. After culture, the supernatants were then removed without disturbing the cell pellets and stored at -20°C until required for cytokine analysis by means of ELISA or time-resolved fluorometry.

Cytokine protein detection

Cytokines in cell-culture supernatants were quantified by using sandwich-type time-resolved fluorometry (IL-6, IL-10, IL-13, and IFN- γ) or by using a commercial ELISA (IL-12 p70), as previously described.¹² The limit of detection was 3 pg/mL for all cytokines.

mRNA quantification by means of real-time RT-PCR

mRNA expression was measured for TLR3, TLR4, and myxovirus resistance protein A (MxA), as well as the reference gene ubiquitin-conjugating enzyme E2D2 (UBC).¹³ Total RNA was isolated from cell-culture pellets by using the Qiagen 96 RNeasy kit (Qiagen, Clifton Hill, Australia); reverse transcription was performed with the Omniscript RT kit (Qiagen) with oligo-dT (Promega, Madison, Wis) and RNasin (Promega). cDNA was diluted 1:5 and quantitated by means of real-time PCR with QuantiTect SYBR Green Master Mix (Qiagen) on the ABI PRISM 7900HT (Applied Biosystems, Foster City, Calif). Dissociation curve analysis was used to confirm the specificity of amplification, and relative copy numbers were determined from plasmid or PCR product standard curves. cDNA-specific assays for TLR4, MxA, and UBC were developed in house; a TLR3 QuantiTect Primer assay from Qiagen

was used, with extension temperatures reduced to 62°C because the product had a melting temperature of 72°C . TLR3, TLR4, and MxA mRNA copy numbers were normalized against UBC copy numbers for each cDNA sample.

Surface and intracellular receptor expression (FACS)

TLR2, TLR3, and TLR4 expression on monocytes (CD14^+), NK cells (CD56^+), myeloid dendritic cells (mDCs; $\text{HLADR}^+\text{CD11c}^+$), and plasmacytoid dendritic cells (pDCs; CD123^+) was studied by using biotinylated anti-human TLR2 (clone TL2.1, 1:50), TLR3 (clone TLR3.7, 1:50), or TLR4 (clone HTA125, 1:50; eBioscience, San Diego, Calif) as primary antibodies, followed by streptavidin conjugated to APCs (1:50, eBioscience) as the secondary antibody. All samples were incubated with TLR or isotype control antibody for 30 minutes at 4°C . Cells were first fixed and permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen, San Diego, Calif) for 30 minutes on ice before staining to detect intracellular TLR3. Flow cytometry was performed on a 6-color laser BD LSR2 flow cytometer (BD Pharmingen) and analyzed with FlowJo software (Tree Star, Ashland, Ore).

Clinical assessment at 12 months

Infants were clinically evaluated at 12 months of age by means of a detailed history and examination by the same pediatric allergist (Dr Susan Prescott). The main allergic outcomes were atopic dermatitis,¹⁴ IgE-mediated food allergy, and sensitization (determined based on the presence of a positive SPT response to allergen extracts [egg, milk, peanut, house dust mite, and cat; Hollister-Stier Laboratories, Spokane, Wash] and a histamine control). A wheal diameter of 3 mm or larger was considered positive. Parents also completed prospective diary cards for infections (chest colds and upper respiratory tract, urinary, gastrointestinal, and other infections) and allergy symptoms, although the limitations of these data are recognized.

Statistical analysis

Groups were compared with the Student *t* test, and data were expressed as geometric means and 95% CIs. Linear regression was used to assess weighted effects (eg, relative influence of maternal allergy or pet exposure) on the dependent variable (eg, neonatal TLR responses) where the dependent variable was normally distributed or normalized by means of logarithmic transformation (assessed by using the Kolmogorov-Smirnov test before inclusion in the linear model). Correlations were assessed by using Spearman or Kendall τ tests. Logistic regression was used to examine the relationship between possible predictors (eg, maternal allergy, immune parameters, and pets) on binary outcome measures (eg, infant allergic outcomes).

RESULTS

Population characteristics

Of the 169 pregnant women initially recruited into the study, 141 mother-baby pairs were followed for 12 months to assess infant allergic outcomes, and 111 had sufficient volumes of cord blood for the immune function studies. The resulting cohort included 71 allergic mothers and 70 mothers with no history of disease or allergic sensitization. At 12 months of age, 38 (27%) infants had diagnosed allergic disease (and 103 infants did not), as shown in Table I. Notably, infants who had allergic disease were more likely to have a pet in the home ($P = .046$).

Effect of maternal allergy on neonatal TLR-mediated responses

Neonatal responses were compared by maternal allergic status as a measure of infant allergy risk. There was no evidence that high-risk infants (with maternal allergy, $n = 59$) had impaired

TABLE I. Characteristics of the study population

	Nonallergic at 1 y of age (n = 103)	Allergic disease diagnosed by 1 y of age (n = 38)	P value
Maternal allergy, no. (%)	54/103 (52.4%)	17/38 (44.7%)	.55
Infant sex, no. (%)			
Male	61/103 (59%)	19/38 (50%)	.34
Female	42/103 (41%)	19/38 (50%)	
Neonatal growth parameters			
Birth weight (kg [SE])	3.435 (0.044)	3.541 (0.056)	.26
Birth length (cm [SE])	50.1 (0.24)	50.0 (0.40)	.25
Mode of delivery, no. (%)			
Vaginal	29/103 (28.2%)	14/38 (36.8%)	.09
Induced vaginal	16/103 (15.5%)	11/38 (28.9%)	
Elective cesarean	45/103 (43.7%)	9/38 (23.7%)	
Emergency cesarean	13/103 (12.6%)	4/38 (10.5%)	
Day-care attendance, no. (%)	96/100 (96.0%)	32/38 (84.2%)	.027
Pets, no. (%)	31/103 (30.1%)	19/38 (50.0%)	.046
Infant feeding			
Ever breastfed, no. (%)	99/103 (96%)	34/38 (90%)	.36
Formula started (age $\pm$ SE)	4.1 $\pm$ 0.36 mo	4.3 $\pm$ 0.55 mo	.71
Solid foods (age $\pm$ SE)	5.31 $\pm$ 0.09 mo	5.31 $\pm$ 0.13 mo	.98
Allergic outcomes at 1 y of age			
Sensitized, no. (%) (positive skin test response to any allergen)	8/92 (8.7%)	18/38 (40.6%)	.001
Atopic dermatitis, no. (%)	0 (%)	34/37 (91.9%)	.001
Food symptoms, no. (%)			
Non-IgE-associated symptom*	3/98 (3.1%)	5/32 (15.6)	.022
IgE-mediated reactions	0 (0%)	3/38 (7.9%)	.001
Other outcomes			
Wheeze, no. (%)	24/103 (23.3%)	12/38 (31.6%)	.38
Wheeze without cold, no. (%)	6/103 (5.8%)	3/37 (8.1%)	.70
Responsive to salbutamol, no. (%)	4/103 (3.9%)	1/36 (2.8%)	1.00

*Non-IgE-mediated food symptoms include gastrointestinal disturbance, a flare of atopic dermatitis in the absence of sensitization, or both.

responses to any of the TLR ligands. Rather, high-risk infants had higher responses compared with low-risk infants ($n = 52$), in particular significantly higher IFN- γ responses to TLR2, TLR3, and TLR4 (Fig 1, A) and IL-12 responses to TLR3 and TLR4 (Fig 1, B). IL-6 responses to TLR3 (Fig 1, D) and IL-10 responses to TLR9 activation were also increased in newborns of allergic mothers (Fig 1, E). Maternal allergy was not associated with any significant differences in TNF- α responses (Fig 1, C) or MxA mRNA expression as a measure of type 1 interferon response (data not shown).

Neonatal TLR-mediated responses as a predictor of allergy at 1 year

Next, neonatal responses were compared according to diagnosed allergic disease in the first year of life.

Infants with diagnosed allergic disease at 1 year of age ($n = 32$) produced significantly higher TNF- α responses to TLR3, TLR4, and TLR5 and significantly higher IL-6 responses to TLR2, TLR4, and TLR 5 at birth than those without subsequent disease ($n = 75$, Fig 2). There were similar trends for IL-10 (Fig 2). Expression of interferon-related MxA mRNA was significantly higher in the allergic group ($P = .032$, data not shown), with a similar trend after TLR5 activation ($P = .09$, data not shown).

When infants were compared on the basis of sensitization (a positive SPT response to any allergen at 12 months of age), the same pattern was seen, with higher TNF- α and IL-6 responses to TLR2, TLR3, TLR4, and TLR5 in the SPT response-positive group compared with the SPT response-negative group, as shown in Table II.

Neonatal TLR3 and TLR4 expression (mRNA) in allergic and nonallergic groups

Expression of TLR3 and TLR4 mRNA was assessed at baseline (without stimulation) and after activation with the corresponding ligand. TLR3 expression was less than the detection limit on most of these cord blood samples. There were no differences between either basal or stimulated TLR4 expressions when groups were compared based on either maternal allergy (Fig 3, A) or subsequent infant allergy (Fig 3, B). TLR4 mRNA expression was downregulated after stimulation with LPS in both groups, but this was more significant in the nonallergic groups (infant or maternal allergy), as shown in Fig 3. Baseline expression of TLR4 mRNA was positively correlated with TLR4-mediated IL-10 responses ($r = 0.32$, $P = .04$) but negatively correlated with IFN- γ responses. Only IL-6 responses were correlated with the LPS-stimulated expression of TLR4 mRNA ($r = 0.21$, $P = .03$).

Collectively, these findings suggest that the pattern of cytokine response might be influenced by the level of receptor expression but that variations in receptor expression (Fig 3) do not account for the differences in cytokine production in the allergic and non-allergic groups (Figs 1 and 2).

TLR surface expression and TLR-induced cytokine production

In a subgroup of high versus low responders, we assessed the relationship between surface TLR expression (detected by means of FACS) on different cell populations and cytokine production in response to TLR ligation. This approach was used because it was

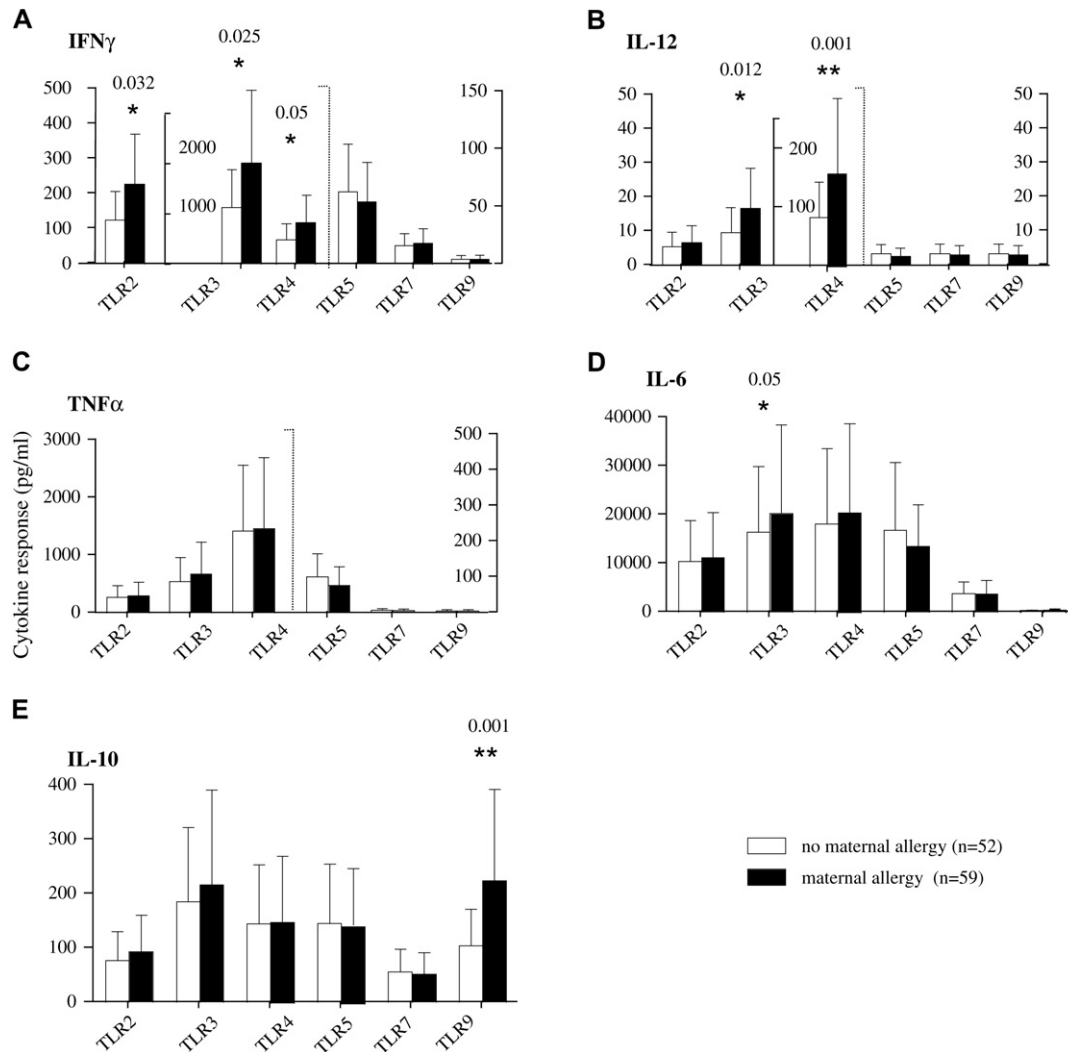


FIG 1. A-E, The relationship between maternal allergy and neonatal TLR responses. Cord blood mononuclear cell cytokine responses after TLR activation (as per the Methods section) are shown for neonates with maternal allergy (solid bars, $n = 59$) and those without maternal allergy (open bars, $n = 52$). Levels are displayed as geometric means and 95% CIs derived for log-transformed data.

not logistically possible to perform flow cytometry on sufficient numbers of the cohort to allow comparison of these measures according to maternal allergy or infant outcomes. Instead, we randomly selected neonates with high-level cytokine responses (>75th percentile) versus low-level responses (<25th percentile), as shown for TLR2, TLR3, and TLR4 in Fig 4, A. We then examined whether these functional differences were related to variations in receptor expression.

There was a significant correlation between TLR4 mRNA (detected in mixed mononuclear cell pellets) and TLR4 surface expression (mean fluorescence intensity [MFI]) on mDCs ($r = 0.75$, $P = .04$) and NK cells ($r = 0.82$, $P = .02$) but not monocytes ($r = -0.17$, $P = .71$).

Although TLR2 was detected predominantly on monocytes and mDCs, it was also detected on pDCs and NK cells (Fig 4, B and C). A representative flow cytometric plot is shown in Fig E1 (available in the Online Repository at www.jacionline.org). There were significant correlations between TLR2 expression (MFI) on pDCs and cytokine production in response

to TLR2 activation, including TNF- α ($r = 0.72$, $P = .020$), IL-10 ($r = 0.93$, $P < .001$), and IL-6 ($r = 0.69$, $P = .026$) production. Although the significance of this is unclear, to our knowledge, this is the first evidence that TLR2 is expressed on neonatal pDCs, albeit at low levels, and that this is strongly associated with the variation in functional (cytokine) responses to TLR2 activation (Fig 4, C). Individual data on the percentage of TLR2 expression within each population is provided in Table E1 (available in the Online Repository at www.jacionline.org).

TLR3 was mainly detected on mDCs (Fig 4, B and C) but also detected at low levels on neonatal NK cells. There were no significant correlations between TLR3 expression and TLR3-mediated cytokine responses. TLR4 was highly expressed on monocytes and to a lesser extent on mDCs, although not on pDCs (Fig 4, B and C). The percentage of mDCs that expressed TLR4 (but not MFI) was correlated with the production of TNF- α ($r = 0.714$, $P = .020$) and IL-12 ($r = 0.58$, $P = .12$).

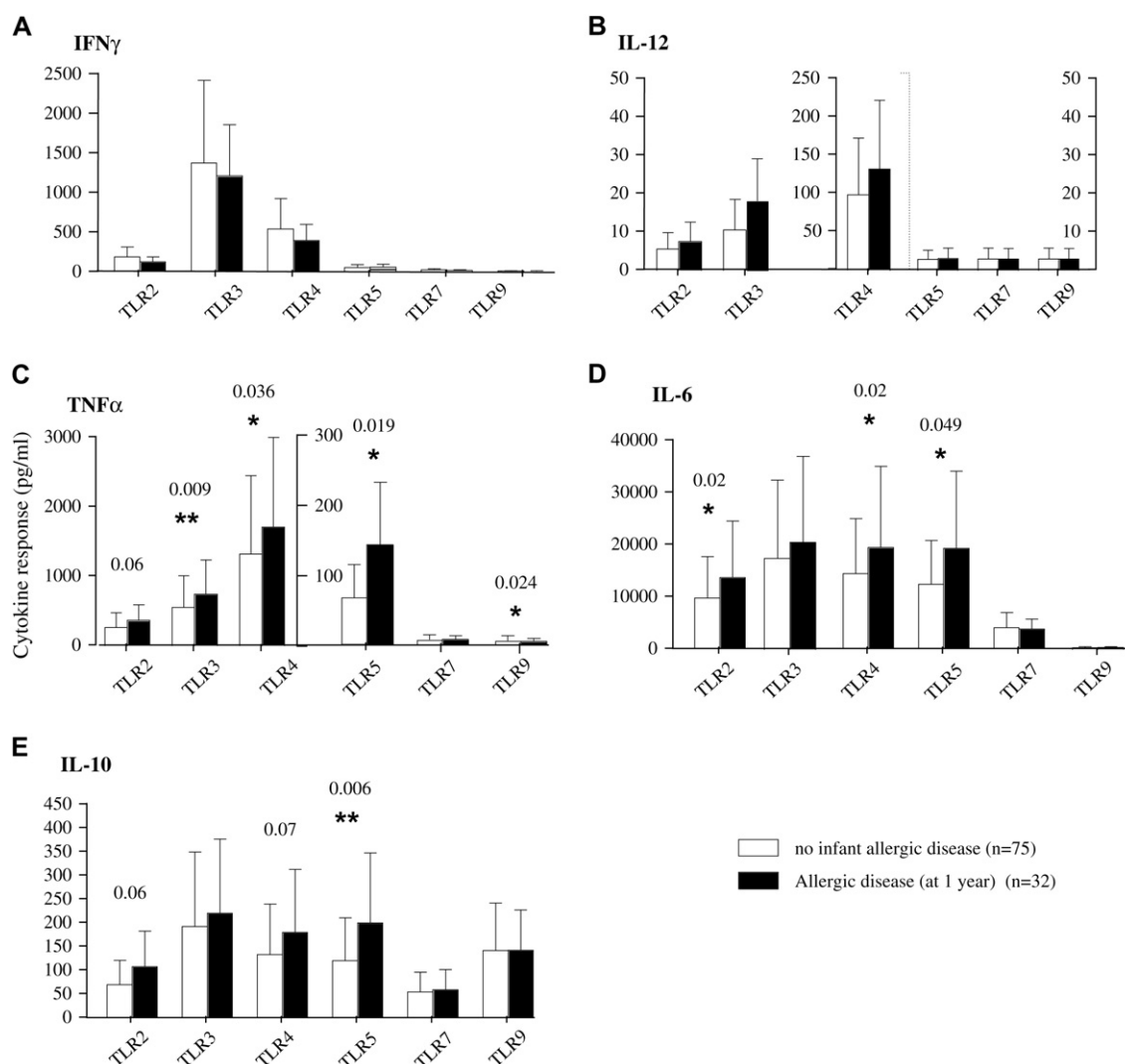


FIG 2. A-E, The relationship between neonatal TLR responses and subsequent infant allergic disease. Neonatal cytokine responses to TLR activation are shown (geometric mean + 95% CI) for those with subsequent allergic disease at 1 year of age (solid bars, n = 32) and those without maternal allergy (open bars, n = 75).

Polyclonal T-cell responses

Consistent with numerous previous reports,¹⁵⁻²⁰ neonates who subsequently had allergic disease (n = 32) had lower T_H1 IFN- γ ($P = .034$) and IL-13 ($P = .017$) responses to PHA mitogen (see Fig E2 in the Online Repository at www.jacionline.org). There were no significant differences in other (IL-10, IL-13, TNF- α , or IL-6) PHA-induced polyclonal responses.

Effect of maternal exposure to pets on neonatal TLR responses

Babies born to mothers who had pets in the home had significantly increased responses to all TLR ligands for IFN- γ (Fig 5). This included higher IFN- γ responses to TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9. There were no differences in the production of other innate cytokines (TNF- α , IL-6, IL-12, or IL-10).

The observed relationship between maternal allergy and TLR-mediated IL-12 and IFN- γ responses remained after adjusting

for the effects of exposure to pets in pregnancy in a linear regression model (see Table E2 in the Online Repository at www.jacionline.org). The same was true of IL-10 responses to TLR9 (adjusted $\beta = .29$; 95% CI, 0.26-1.33; $P = .004$; data not shown).

As already indicated above, infant allergy was not associated with significant differences in type 1 cytokine responses to TLR (Fig 2, A and B), and this did not change after allowing for the effects of maternal allergy and pets (see Table E2 in the Online Repository). Thus maternal allergy (rather than subsequent infant allergy or pets) was the major factor determining neonatal IL-12 responses.

Most families with pets had cats, dogs, or both, which had been there for a number of years by the time the child was seen at 12 months of age (on average, 5.51 years [SE $\pm$ 1.01 years, n = 16] for cats and 5.11 years [SE $\pm$ 0.64 years, n = 29] for dogs. Some families also had rabbits (for 2.52 $\pm$ 1.29 years; n = 3) or birds (2.02 $\pm$ 0.64 years; n = 10). Very few families got new pets during pregnancy or in the first year of life.

TABLE II. Comparison of neonatal TLR responses in children with (n = 20) and without (n = 91) allergen sensitization at 1 year of age

	Geometric mean (95% CIs)														
	IL-6 (pg/mL)			TNF-α (pg/mL)			IL-10 (pg/mL)			IFN-γ (pg/mL)			IL-12p70 (pg/mL)		
	SPT –	SPT +	P value	SPT –	SPT +	P value	SPT –	SPT +	P value	SPT –	SPT +	P value	SPT –	SPT +	P value
TLR2	9766 (8270-11,535)	16,813 (13,888-20,353)	.035	446 (371-536)	783 (544-1126)	.015	226 (185-276)	259 (197-341)	.014	157 (111-223)	210 (85-521)	.520	5.6 (4.5-7.1)	8.2 (4.9-13.6)	.03
TLR3	17,597 (15,454-20,038)	23,496 (19,980-27,628)	.07	556 (462-669)	869 (584-1292)	.035	190 (154-235)	237 (180-311)	.362	1289 (965-1721)	1725 (901-3301)	.425	12.1 (9.3-15.8)	16.8 (9.5-29.7)	.298
TLR4	14,722 (11,276-19,222)	23,957 (21,506-26,683)	.007	1347 (1155-1570)	1992 (1475-2691)	.038	139 (112-171)	179 (133-242)	.288	496 (355-691)	516 (202-1316)	.926	105 (82-135)	140 (86-227)	.344
TLR5	12,739 (8994-18,043)	22,007 (17,560-27,579)	.039	70 (50-99)	171 (90-324)	.018	126 (97-164)	211 (158-281)	.05	47 (33-67)	93 (45-190)	.343	2.9 (2.5-3.4)	3.2 (2.9-3.6)	.464
TLR7	3802 (2894-4994)	4387 (1414-13611)	.195	5.3 (4.4-6.3)	7.4 (3.7-14.9)	.181	53 (43-66)	60 (39-94)	.627	16 (12-22)	23 (7-76)	.120	3.0 (2.8-3.2)	3.4 (2.9-4.0)	.024
TLR9	252 (178-357)	468 (144-1519)	.187	4.5 (3.9-5.3)	3.6 (2.8-4.7)	.330	139 (100-192)	149 (70-317)	.870	4 (3-5)	5 (3-7)	.435	2.9 (2.5-3.4)	3.2 (2.9-3.6)	.554

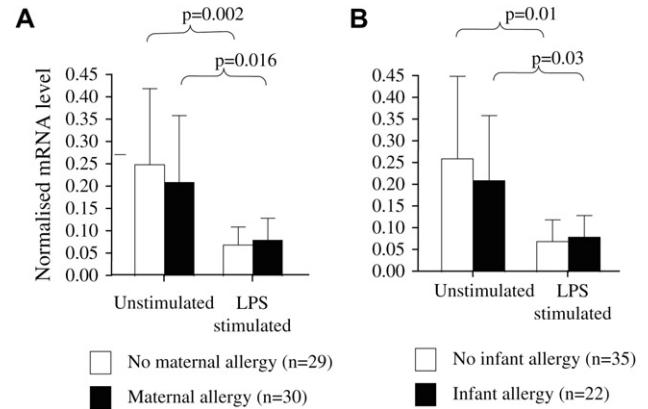


FIG 3. Comparison of TLR4 expression in allergic and nonallergic groups. The expression of neonatal TLR4 mRNA (geometric mean + 95% CI) is shown at rest (baseline unstimulated) and after LPS stimulation. Levels are compared according to maternal allergy (**A**) and infant allergy (**B**).

Predictors of infant allergic disease

Logistic regression was used to determine the main predictors of infant allergy (with allergic outcome as the dependent variable). In this population “household pets” was the strongest predictor of subsequent disease (OR, 3.0; 95% CI, 1.3-7.4; $P = .012$). This effect was stronger than maternal allergy or any of the immunologic or environmental associations (see below).

Children regularly attending day care were less likely to have allergic disease (OR, 0.22; 95% CI, 0.06-0.84; $P = .026$), as were children who had recurrent chest infections (OR, 0.71; 95% CI, 0.51-0.98). Notably, children with recurrent chest colds had lower TNF- α responses to TLR3 ($P = .045$) and TLR2 ($P = .066$). However, the majority of the observed relationships between neonatal TNF- α (and IL-6) responses and infant allergic disease (Fig 2) remained statistically significant after allowing for the effect of pets, maternal allergy, day care, and infection history (see Table E3 in the Online Repository at www.jacionline.org), although these effects were weak relative to the other predictors (ie, pets and day care).

DISCUSSION

This study demonstrates that infant allergic disease is associated with presymptomatic differences in TLR function, with relative hyperresponsiveness to TLR ligands. These differences were apparent at birth before colonization and imply genetic influences, modulating environmental influences, or both *in utero*. Furthermore, higher IL-6 and TNF- α responses to TLR4-mediated responses in the allergic group were not associated with differences in TLR4 (mRNA) expression, which, if anything, tended to be lower or equivalent in these children before stimulation. This might suggest differences in downstream intracellular events that drive cytokine production in subsequently allergic children. We also noted that maternal allergy was significantly associated with higher neonatal TLR responses, particularly type 1 cytokine responses (IL-12 and IFN- γ) to TLR stimulation. It could be argued that this is an appropriate adaptive neonatal response poised to suppress the allergic propensity (conferred by the maternal environment, maternal allergy, or both) on contact with the external microbial environment. Because these infants were all born in an environment in which microbial burden is typically low

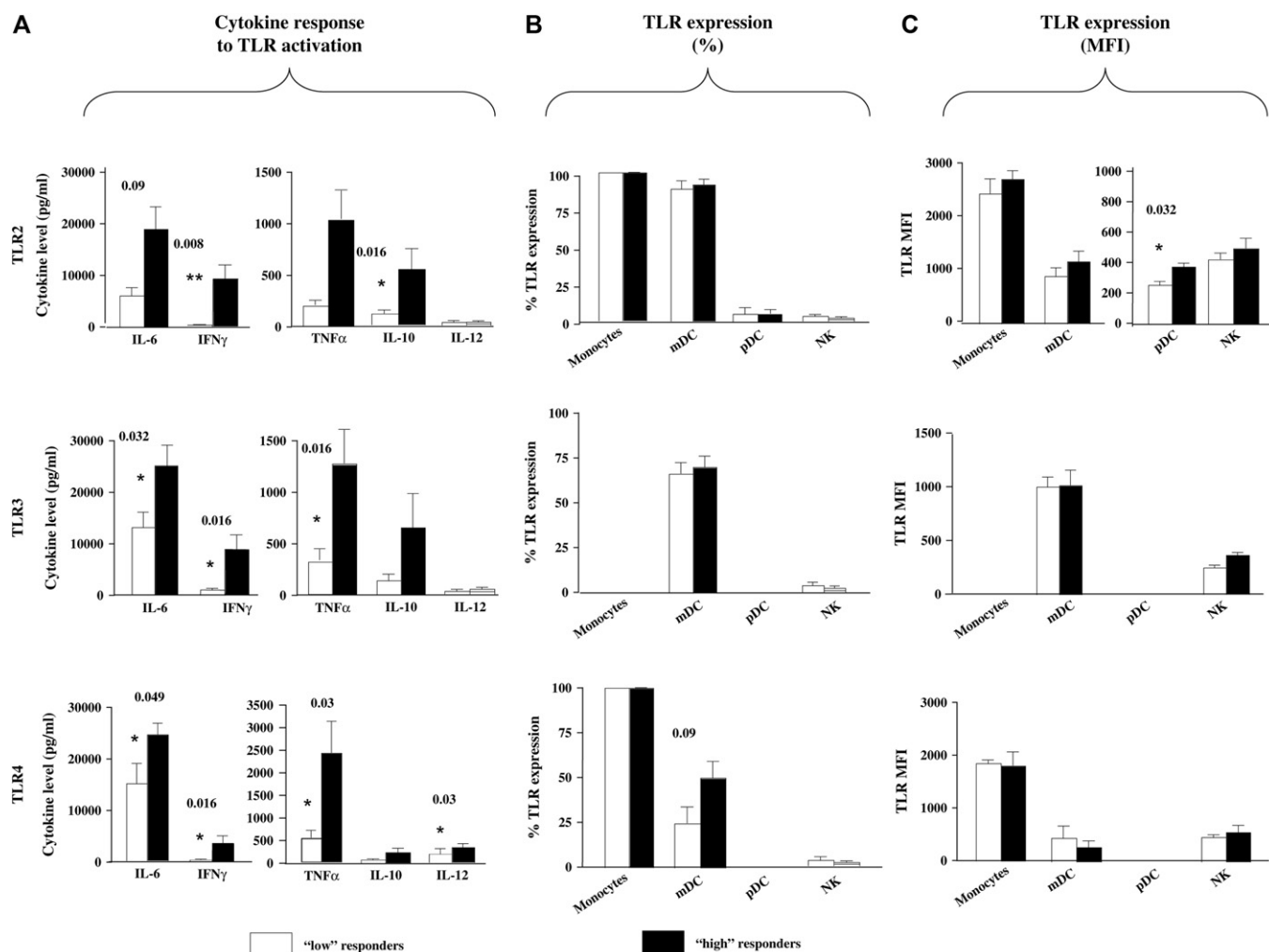


FIG 4. Comparison of TLR expression (detected by FACS) in high- versus low-cytokine responders to TLR ligation. Neonates were randomly selected for FACS studies on the basis of their responder phenotype, and high responders (>75% percentile; solid bars, $n = 5$) were compared with low responders (<25% percentile; open bars, $n = 5$). Cytokine responses are shown (A), together with the TLR expression on each cell population as both percentage of cells expressing each TLR in comparison with negative control, which was set at 0% (B), and the MFI for the TLR⁺ cells in each subset (C).

(suburban Perth, Australia), postnatal exposure might be insufficient to suppress the allergic propensity through these pathways in many infants. It is difficult to determine the relative contribution of paternal atopic heredity in this population because paternal allergy testing was not performed as a result of logistic limitations.

The findings of our study challenge the notion that allergic disease is associated with a simple insufficiency of TLR responsiveness in early life. They also challenge one previous study that noted maternal allergy ($n = 9$) was associated with decreased neonatal TLR2 responses relative to those of children born to nonallergic women ($n = 10$).⁷ However, that study was substantially smaller and did not examine clinical outcomes. Similarly, the only other study to report TLR expression in relation to maternal allergy noted reduced mRNA expression (or TLR2 and TLR4) in cord blood⁸ but did not examine functional responses or allergic outcomes.

Variations in the timing and duration of microbial exposure might provide the key to understanding apparent inconsistencies

with the hygiene hypothesis. Although acute infections increase TLR function for host defence, it is logical that chronic microbial exposure leads to downregulation of these pathways. In the early postnatal period, there is some evidence that microbial burden downregulates TLR function. Although TLRs are highly expressed on the intestinal surface in the neonatal period, expression and function of these receptors are rapidly downregulated during normal colonization,^{21,22} protecting the neonate from excessive inflammatory responses to colonizing commensal flora. In later childhood there is also some evidence that chronic exposure to high levels of bacterial endotoxin (in farmer's children) is associated with less allergic disease and with downregulated TLR-mediated (IFN- γ , IL-10, TNF- α , and IL-12) cytokine responses.²³ In this context it can be expected that hyperresponsiveness might reflect reduced, rather than increased, chronic microbial exposure.

As a possible surrogate marker of higher endotoxin exposure, the presence of household pets was associated with a generalized increase in TLR responses (TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, and TLR9). The mechanism and significance of this

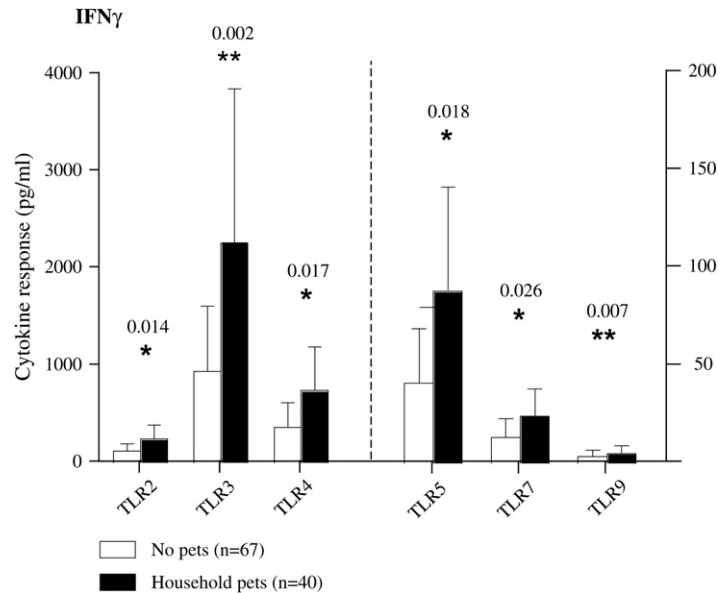


FIG 5. The relationship between neonatal TLR responses and the presence of pets in the maternal home. Neonatal IFN- γ responses to TLR activation are shown (geometric mean + 95% CI) for those with pets in the maternal home (solid bars, n = 40) and those without pets (open bars, n = 67).

association are uncertain, and endotoxin levels were not measured in this study. If this relationship does represent an endotoxin-mediated effect, this would not support the hypothesis that chronic endotoxin exposure downregulates TLR responses. However, the level or dose is likely to be an important factor determining this relationship because pet exposure is in no way likely to reproduce the microbial burden of traditional environments. Consistent with other studies,^{24,25} we also noted a protective relationship between day-care attendance and allergic outcomes. There was also a protective effect of chest colds, which were also associated with lower neonatal TNF- α responses to TLR2 and TLR3 ligands. This could suggest another hypothesis for the lower TLR responses seen in the nonallergic infants, namely that this group had early susceptibility to infection that inhibited the development of allergic propensity. However, because infection history was based on parental reporting, this aspect of the findings must be interpreted with caution.

In summary, this is the first study to show that allergic disease is preceded by early hyperresponsiveness to innate immune activation, apparently without corresponding maturation of effector T-cell function (as assessed by PHA responses). Other environmental factors have also been shown to alter TLR function, including maternal smoking in pregnancy,²⁶ breast-feeding,²⁷ and dietary polyunsaturated fatty acids.²⁸ Thus early TLR function is likely to reflect complex genetic and environmental interactions. Notably, the effects of microbial exposure are likely to depend on the timing, duration, dose, and type of microorganisms involved. There is now evidence that these processes might be initiated antenatally. Assessment of postnatal responses is necessary (and underway) in these infants to determine how TLR responses are modified during early colonization and environment-driven immune maturation. This will help determine the normal ontogeny of these pathways and potential differences in allergic children. Long-term follow-up of this population will also allow us to determine whether the functional differences in neonatal innate immunity observed in relation to early allergic outcomes are

also evident in relation to subsequent manifestations of allergic disease.

We thank the obstetricians, midwives, and volunteers who assisted in this study.

Key messages

- This study provides preliminary evidence that neonates who progress to allergic disease have presymptomatic differences in innate immune function.
- Further studies are needed to determine how these TLR-mediated responses mature and influence allergen-specific responses in the postnatal period.

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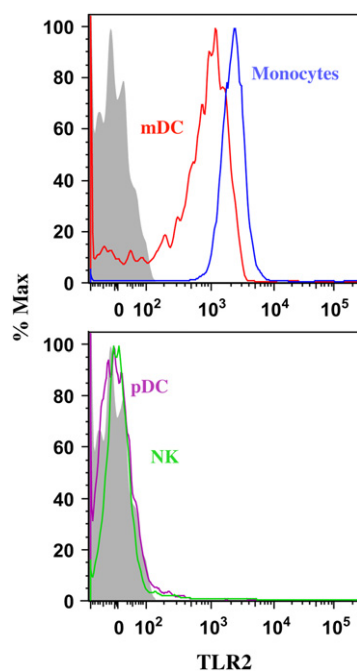


FIG E1. A representative example of high-responder TLR2 expression in different cell types. The relative intensity of TLR2 expression in monocytes (*blue*), mDCs (*red*), pDCs (*purple*), and NK cells (*green*) in relation to one another and to negative unstained control (*shaded*) is shown. Results illustrate almost all monocytes to be TLR2⁺ (99.9% total cells), followed by mDCs (87.5%) and a smaller but significant detection of TLR2 in pDCs (1.64%) and NK cells (2.37%).

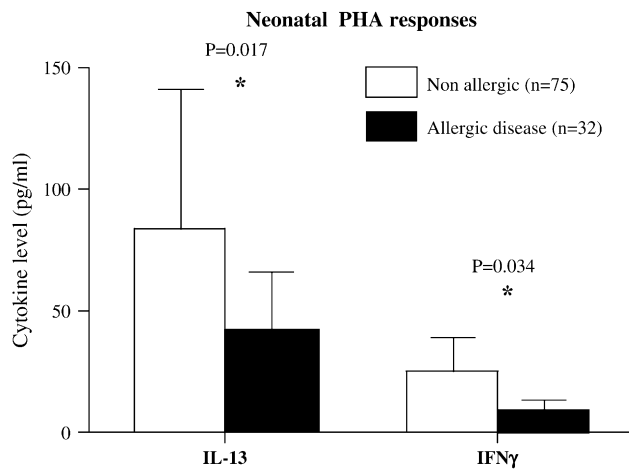


FIG E2. The relationship between neonatal polyclonal T-cell responses and subsequent allergic disease. Neonatal T_H1 (IFN- γ) and T_H2 (IL-13) responses to polyclonal activation with PHA are shown for neonates with subsequent allergic disease (*solid bars*, n = 32) and those without allergy (*open bars*, n = 75). Levels are displayed as geometric means and 95% CIs. Significant differences between the groups are shown.

TABLE E1. Individual TLR2 flow cytometric data for neonatal samples used to compare high-cytokine responders with low-cytokine responders

	Monocytes		mDCs		pDCs		NK cells	
	Total (%) [*]	TLR2 ⁺ (%) [†]	Total (%)	TLR2 ⁺ (%)	Total (%)	TLR2 ⁺ (%)	Total (%)	TLR2 ⁺ (%)
High responders								
1	34.2	99.9	10.3	99	3.07	0.99	9.16	1.82
5	7.88	100	8.66	99.5	1.86	0.49	14.1	0.42
6	7.59	99.9	13.2	87.5	1.68	1.64	15.2	2.37
7	17.7	100	11.6	82.2	2.38	0.84	9.83	0.83
9	18.4	99.9	9.73	98.5	3.01	0.64	18.7	0.45
Average	17.15	99.94	10.70	93.34	2.4	0.92	13.40	1.18
Count	5	5	5	5	5	5	5	5
SD	10.84	0.05	1.76	7.98	0.64	0.45	3.95	0.87
SE	5.42	0.03	0.88	3.99	0.32	0.22	1.98	0.44
Low responders								
2	13.6	100	16.6	99.3	2.31	2.83	8.21	0.66
3	9.54	100	11.4	98.6	1.92	1.32	5.74	0.8
4	15.2	99.9	2.29	88.5	2.55	3.45	4.76	4.83
8	17.5	99.9	5.09	70.6	2.61	0.49	5.87	1.49
10	13.3	100	9.78	95.2	1.53	3.45	4.62	0.82
Average	13.83	99.96	9.03	90.44	1.73	2.31	5.84	1.72
Count	5	5	5	5	5	5	5	5
SD	2.92	0.05	5.58	11.89	0.92	1.34	1.44	1.77
SE	1.46	0.03	2.79	5.94	0.46	0.67	0.72	0.88

*Percentage of total cell population.

†Percentage of each population that are TLR2⁺.

TABLE E2. Independent effects of maternal allergy, infant allergy, and household pets on TLR-mediated IL-12 and IFN- γ responses

	TLR2		TLR3		TLR4	
	β (95% CI)	<i>P</i> value	β (95% CI)	<i>P</i> value	β (95% CI)	<i>P</i> value
IL-12						
Maternal allergy	0.10 (−0.23 to 0.65)	.36	0.28 (0.14 to 1.10)	.012	0.32 (0.18 to 1.08)	.007
Household pets	−0.004 (−0.47 to 0.46)	.97	0.062 (−0.38 to 0.66)	.58	−0.03 (−0.55 to 0.42)	.79
Infant allergy	0.13 (−0.24 to 0.79)	.29	0.18 (−0.12 to 1.04)	.12	0.14 (−0.21 to 0.87)	.23
IFN- γ						
Maternal allergy	0.20 (0.15 to 1.31)	.045	0.27 (0.22 to 1.27)	.006	0.21 (0.05 to 1.32)	.034
Household pets	0.28 (0.26 to 1.66)	.008	0.33 (0.37 to 1.51)	.001	0.21 (0.01 to 1.38)	.046
Infant allergy	−0.15 (−1.38 to 0.20)	.14	−0.04 (−0.76 to 0.52)	.71	−0.07 (−1.02 to 0.52)	.53

Multivariate linear regression analysis for TLR-mediated IL-12 and IFN- γ responses.

TABLE E3. Relationship between infant allergic disease and neonatal TLR responses after allowing for potential effects of maternal allergy, day-care attendance, number of chest colds, and the presence of pets in the home

	TNF- α		IL-10		IL-6	
	β (95% CI)	<i>P</i> value	β (95% CI)	<i>P</i> value	β (95% CI)	<i>P</i> value
TLR2	1.001 (1.000-1.002)	.035	1.001 (0.999-1.003)	.490	1.000 (1.000-1.000)	.015
TLR3	1.001 (1.000-1.002)	.010	1.001 (0.999-1.004)	.347	1.000 (1.000-1.000)	.129
TLR4	1.001 (1.000-1.002)	.017	1.001 (0.999-1.003)	.315	1.000 (1.000-1.000)	.017
TLR5	1.002 (0.999-1.004)	.160	1.002 (1.000-1.006)	.08	1.000 (1.000-1.000)	.062

Logistic regression analysis (with infant allergic disease as the dependent variable).