

Differences in innate immune function between allergic and nonallergic children: New insights into immune ontogeny

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Background: Microbial products are of central interest in the modulation of allergic propensity.

Objective: We sought to explore whether allergic children show differences in microbial Toll-like receptor (TLR)–mediated responses over their first 5 years of life.

Methods: Mononuclear cells isolated from 35 allergic and 35 nonallergic children at birth and 1, 2.5, and 5 years of age were stimulated with TLR2–TLR9 ligands to study innate immune function and with allergens or mitogen to assess adaptive T-cell responses. Cytokine production was measured by using Luminex multiplexing technology.

Results: Nonallergic children show progressive and significant age-related increases in innate cytokine responses (IL-1 β , IL-6, TNF- α , and IL-10) to virtually all TLR ligands. This innate maturation corresponds with a parallel increase in adaptive T_H1 (IFN- γ) responses to allergens and mitogens. In contrast, allergic children show exaggerated innate responses at birth ($P < .01$) but a relative decrease with age thereafter, so that by age 5 years, TLR responses are attenuated compared with those seen in nonallergic subjects ($P < .05$). This early hyperresponsiveness in allergic subjects fails to translate to a corresponding maturation of T_H1 function, which remains attenuated relative to that seen in nonallergic subjects but is associated with a characteristic age-dependent increase in allergen-specific T_H2 responses ($P < .01$).

Conclusion: Our findings suggest significant differences in the developmental trajectory of innate immune function in children

with allergic disease that might contribute to the recognized differences in postnatal adaptive T-cell immunity. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Toll-like receptor, ontogeny, innate immunity, allergic disease, children

Despite intense interest in the role of decreasing microbial burden in the dramatic increase in allergic disease and other immune disorders,¹ the normal development of microbial recognition pathways has not yet been documented. This is the first longitudinal study to map the functional ontogeny of key microbial pattern-recognition receptors in childhood, notably the Toll-like receptors (TLRs). It specifically examines the emerging hypothesis that children with allergic disease have altered responsiveness to microbial components, which might lead to abnormal patterns of immune maturation that underpin disease predisposition.

This study builds on our landmark study published in the *Lancet* in 1999,² which established the current paradigm that neonatal responses must undergo environment-driven T_H1 maturation in the postnatal period. Postnatal suppression of T_H2 propensity has been attributed to exposure to an increasingly diverse microbial environment during the first years of life.² Until now, there has been no clear evidence that the failure of T_H2 suppression in allergic children is related to an underlying disorder of innate microbial recognition pathways. We speculated that deviations in innate TLR-mediated responses are responsible for the differences in allergic predisposition, which appear more likely to emerge in an environment with low microbial burden.

In recent studies we have already identified presymptomatic differences in TLR function in neonates who subsequently had allergic disease compared with their nonallergic counterparts.³ Specifically, subsequent allergic disease was associated with increased neonatal TNF- α responses to TLR3, TLR4, and TLR5 ligands and increased IL-6 responses to TLR2, TLR4, and TLR5 ligands. Despite this, neonatal T-cell IFN- γ responses to allergens and mitogens remained attenuated compared with those seen in the nonallergic group, as consistently observed in many previous studies,^{4,5} providing new evidence of discordance between the innate and adaptive arms of the neonatal immune response in allergic children. These perinatal differences in microbial responses³ also highlight the emerging role of antenatal events.^{6,7} In this new study we aim to examine the postnatal consequences of this perinatal immune dysregulation in an independent cohort in the first comprehensive longitudinal analysis of microbial recognition pathways over the first 5 years of life.

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Abbreviations used

APC: Antigen-presenting cell
 mDC: myeloid dendritic cell
 NK: Natural killer
 pDC: Plasmacytoid dendritic cell
 SPT: Skin prick test
 TLR: Toll-like receptor

METHODS**Study design**

This was a case-control study of 35 allergic and 35 nonallergic children randomly selected from 739 children enrolled in prospective birth cohorts designed to examine allergy pathogenesis. The children had to meet the clinical definitions for respective allergic status (below), have blood samples collected at each time point (birth and 1, 2.5, and 5 years of age), and have had full clinical assessments for allergic disease over this follow-up period to be eligible for selection.

Clinical definitions

Children were defined as nonallergic if they had no symptoms of allergic disease and no evidence of allergic sensitization during the first 5 years. The allergic group comprised children who had doctor-diagnosed allergic disease and evidence of allergic sensitization at 1 or more postnatal visits. This was based on a detailed history and examination by the same pediatric allergist (Professor Susan Prescott) evaluated at 1, 2.5, and 5 years of age. A diagnosis of eczema was based on typical skin lesions⁸ responsive to topical steroids. IgE-mediated food allergy was defined as a history of immediate symptoms after contact with food, ingestion of food, or both and a positive skin prick test (SPT) response to that food. Symptoms of acute food allergy included skin reactions (hives, rash, or swelling) and/or respiratory tract symptoms (cough, wheeze, or stridor) and/or gastrointestinal symptoms (abdominal pain, vomiting, or loose stools) and/or cardiovascular symptoms (collapse). Asthma was defined as recurrent wheezing with evidence of responsiveness to bronchodilator therapy.

Sensitization was determined by the presence of a positive SPT response to allergen extracts (egg, milk, peanut, house dust mite, cat, rye grass, and southern grass mix; Hollister-Stier Laboratories, Spokane, Wash) and a histamine control. A wheal diameter of 3 mm or greater was considered positive. Parents completed prospective diary cards for infections (chest colds, upper respiratory tract infections, and urinary, gastrointestinal, and other infections) and allergy symptoms.

Other clinical data

All patients were recruited antenatally from healthy pregnant mothers, aged 18 to 45 years, recruited from private clinics of obstetricians in Perth, Australia. Only term infants were eligible. Maternal allergy was defined by a history of doctor-diagnosed asthma, eczema, or allergic rhinitis and a positive SPT response to 1 or more common allergens. Nonallergic mothers had no history of allergic disease and had negative SPT responses to all tested allergens. Paternal history was based on doctor-diagnosed asthma, eczema, or allergic rhinitis (but SPTs were not performed on fathers for logistic reasons).

Perinatal data were collected directly from the hospital charts. Postnatal data were collected prospectively (diary cards) and included information about other clinical diseases and common exposures (vaccination, infection, day care, diet, and medication use, including antibiotics), as well as the home environment (including carpeting, sibship, and pets). This study has been approved by the Princess Margaret Hospital Ethics Committee.

Blood collection

Cord blood was collected immediately after delivery by means of venipuncture of placental vessels, as previously described.² At 1, 2.5, and 5

years of age, blood samples (up to 5 mL) were taken with parental consent from a vein in the cubital fossa after anesthetization with lidocaine (Emla cream; AstraZeneca, Wilmington, Del) by a qualified phlebotomist. Mononuclear cells were isolated by means of Ficoll-Hypaque gradient centrifugation and cryopreserved (in 7.5% dimethyl sulfoxide) for future use by using established techniques.⁹ Adult cells were collected for comparison from healthy nonallergic volunteers.

Assessment of innate TLR function

Cord blood mononuclear cells and PBMCs were cultured alone or with optimized doses of lipoteichoic acid (1 μ g/mL, TLR2 ligand), poly I:C (50 μ g/mL, TLR3 ligand), *Escherichia coli* LPS (10 ng/mL, TLR4 ligand), flagellin (1 μ g/mL, TLR5 ligand), zymosan (10 μ g/mL, TLR2/6 ligand), Gardiquimod (10 μ g/mL, TLR7/8 ligand), and CpG oligonucleotide (3 μ g/mL, TLR9 ligand), all of which were purchased from InvivoGen (San Diego, Calif).

All cultures were plated in duplicates in 96-well round-bottom plates in 250 μ L of RPMI (Gibco Life Technology, Grand Island, NY) plus non-heat-inactivated 10% FCS (Australian Biosearch, Karrinyup, Australia) and incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. Cells incubated with Gardiquimod and CpG were cultured for 48 hours. Parallel LPS (TLR4) cultures were performed primed with recombinant IFN- γ (10 ng/mL) for 3 hours before the addition of LPS (to detect IL-12). After culture, the supernatants were removed and stored at -20°C until cytokine analysis.

Assessment of adaptive/polyclonal mitogen responses

To assess polyclonal T-cell responses, PBMCs were cultured in AIM-V 2ME media in the presence of PHA (1 μ g/mL) mitogen (HA16; Murex Biotech Ltd, Dartford, United Kingdom) for 48 hours. Allergen-specific responses to house dust mite (10 μ g/mL; kindly donated by the Department of Parasitology, Mahidol University, Bangkok, Thailand) and egg (ovalbumin 100 μ g/mL) were also measured 48 hours after culture.

Cytokine protein detection

Cytokines (IL-1 β , IL-6, IL-10, IL-12, IL-13, TNF- α , and IFN- γ) in cell-culture supernatants were quantified with Luminex Xmap multiplexing technology (Luminex Corp, Austin, Tex). The limit of detection was 3 pg/mL for all cytokines, and all data are shown as increases above unstimulated/baseline responses.

Surface and intracellular TLR expression (FACS)

TLR2, TLR3, TLR4, and TLR9 expression on monocytes (HLA-DR⁺CD14⁺), natural killer (NK) cells (CD16⁺CD56⁺), myeloid dendritic cells (mDCs; Lin1⁻HLA-DR⁺CD11c⁺CD123⁻), and plasmacytoid dendritic cells (pDCs; Lin1⁻HLA-DR⁺CD123⁺CD11c⁻) was studied by means of flow cytometry on a randomly selected subpopulation of 10 infants (5 allergic and 5 nonallergic subjects) by using antibodies directed against human TLR2 conjugated to phycoerythrin (clone TLR2.1, 1:200; eBiosciences, San Diego, Calif), TLR4 conjugated to PEcy7 (clone HTA125, 1:5; eBiosciences), and TLR9 conjugated to antigen-presenting cells (APCs; clone eB72-1665, 1:50; BD Biosciences, Mississauga, Ontario, Canada). Biotinylated anti-human TLR3 (clone TLR104, 1:5; BioLegend, San Diego, Calif) was used as the primary antibody followed by streptavidin conjugated to APCs (1:20, eBiosciences) as the secondary antibody. All samples were incubated with TLR or isotype control for 30 minutes at 4°C. Cells were permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen, San Diego, Calif) for 30 minutes before staining to detect intracellular TLR3 and TLR9. All gates were set by using samples stained with appropriate concentration-matched isotype controls. Flow cytometry was performed on a 6-color laser BD LSR II flow cytometer (BD Biosciences). Fifty thousand events were acquired for mDCs, monocytes, and NK cells, and 250,000 events were acquired for pDCs. The data were analyzed with FlowJo software (Tree Star, Ashland, Ore).

Statistical analysis

Statistical analysis of developmental changes in cytokine response was assessed by using standard 2-stage or summary measures analysis, as previously described.^{10,11} Differences between groups (allergic and nonallergic subjects) at each time point were compared with the Student *t* test on log-transformed data. Individual slopes for each subject were estimated by means of linear regression of the log-transformed cytokine values on age to describe the trajectory of cytokine responses over time. Significant differences from zero slope were determined by using a single-sample *t* test. For example, positive coefficients suggest that cytokine levels increased with age, whereas negative coefficients suggest that cytokine levels decreased with age. Mean differences in the trajectory (slope) of cytokine responses (regression coefficients) between the 2 groups were compared by using 2-sample independent *t* tests.

Linear regression was used to assess possible confounders (eg, the relative influence of maternal allergy, paternal allergy, and infant sex) on the dependent variable (eg, neonatal TLR responses), where normality of the dependent variable was assessed by using QQ plots and Kolmogorov-Smirnov tests before inclusion in the linear model. Logistic regression was used to examine the relationship between possible predictors (immune function) over time on binary outcome measures (eg, infant allergic outcomes). A significance threshold of .05 was used for all tests, unless otherwise stated.

RESULTS

Population characteristics

The characteristics of our study population are shown in Table I. The allergic group had doctor-diagnosed disease (see definitions) at 1 or more of the study time points with evidence of allergen sensitization (positive SPT response), whereas the nonallergic group had no disease or sensitization at any time point. All children had sufficient volumes of blood collected at each of the study time points to perform longitudinal immune function studies. The patterns of disease and sensitization in the allergic group are also shown on Table I. The general characteristics of these groups were similar, with the exceptions of differences in anthropometric measures, including lower birth weight ($P = .01$) and head circumference ($P = .02$) in the allergic group. Difference in anthropometric measures and paternal allergy were examined as potential confounding variables in regression analyses and found not to contribute to the differences in immune function described below or to the risk of allergy development. There was a trend toward more paternal allergy ($P = .05$) in the allergic group but no differences in maternal allergy ($P = .12$).

Ontogeny of innate and adaptive immune function in nonallergic children

Nonallergic children show a progressive and significant age-related increase in TLR-mediated innate cytokine responses, as exemplified by IL-1 β and TNF- α in Fig 1. This apparent maturation was observed with virtually all TLR agonists tested, but the most profound effect was seen with TLR2-induced ($P < .001$), TLR3-induced ($P < .001$), and TLR9-induced ($P < .001$) IL-1 β responses (Fig 1, A) and TLR2-induced ($P < .01$), TLR3-induced ($P < .01$), TLR4-induced ($P < .01$), TLR2/6-induced ($P < .001$), and TLR5-induced ($P < .001$) TNF- α responses (Fig 1, B). Similar patterns were seen for other cytokines, including IL-6 (Table II) and IL-10 (Table III). IL-12 was only consistently detectable in samples primed with IFN- γ and then stimulated with LPS, and this showed the same age-related increase in responsiveness (see Table E1 in this article's Online Repository at www.jacionline.org), which is consistent with previous cross-

TABLE I. Characteristics of the study population

Characteristics	Nonallergic children (n = 35)	Allergic children (n = 35)	Significance, <i>P</i> value
Sex			
Male (%)	43	57	
Female (%)	59	41	.19
Family history of allergy			
Maternal allergy (%)	66	82	.12
Paternal allergy (%)	47	71	.05
Maternal age (SE)	34.7 (0.7)	32.9 (0.7)	.06
Neonatal growth parameters			
Birth weight (g [SE])	3,555 (69)	3,312 (62)	.01
Birth length (cm [SE])	50.3 (0.4)	49.6 (0.3)	.15
Head circumference at birth (cm [SE])	35.3 (0.2)	34.5 (0.2)	.02
Gestation (wk [SE])	39.3 (0.2)	38.8 (0.3)	.20
Parity (SE)	0.7 (0.1)	0.6 (0.1)	.52
Gravidity (SE)	2.3 (0.2)	2.2 (0.2)	.78
Delivery method			
Vaginal delivery (%)	57	71	.28
Cesarean section (%)	43	29	.25
Day care attendance (%)	89	94	.46
Pets in the home (%)	49	34	.24
Infant feeding			
Ever breast-fed (%)	97	97	1.0
Formula started (age in mo $\pm$ SE)	4.6 $\pm$ 0.8	3.3 $\pm$ 0.6	.12
Solid foods started (age in mo $\pm$ SE)	5.1 $\pm$ 0.2	5.1 $\pm$ 0.2	.10
Allergic phenotype			
Any allergic disease, no. (%)	0	35 (100)	<.001
Atopic dermatitis, no. (%)	0	32 (92)	.001
Asthma, no. (%)	0	12 (34)	.004
Food allergy (IgE mediated), no. (%)	0	3 (9)	<.001
Sensitized, no. (%), at any age	0	35 (100)	<.001
Milk	0	3 (9)	<.001
Egg	0	8 (23)	<.001
Peanut	0	4 (11)	<.001
House dust mite	0	32 (91)	<.001
Grass pollens	0	27 (77)	<.001
Animals	0	19 (54)	<.001
Use of asthma medication, no. (%)			
Bronchodilator therapies	0	8 (23)	<.001
Inhaled corticosteroid therapies	0	10 (29)	<.001

Significant differences between the groups were determined by using the Pearson χ^2 test for all categorical data and by using Student *t* tests for continuous data.

sectional studies of IL-12 capacity.¹² With few exceptions, the neonatal TLR responses were generally lower than corresponding adult responses (Fig 1).

Next, we compared this innate developmental trajectory with age-related changes in T-cell adaptive immunity. As described in our previous studies,² nonallergic children show a statistically significant age-dependent increase in adaptive T_H1 immunity (ie, IFN- γ responses to allergens [house dust mite and ovalbumin] and mitogens [PHA]; Fig 2, A). Notably, this T_H1 maturation occurred in parallel with the innate responses to TLR ligands shown in Fig 1. Nonallergic children showed very strong and highly significant correlations between their innate immune development

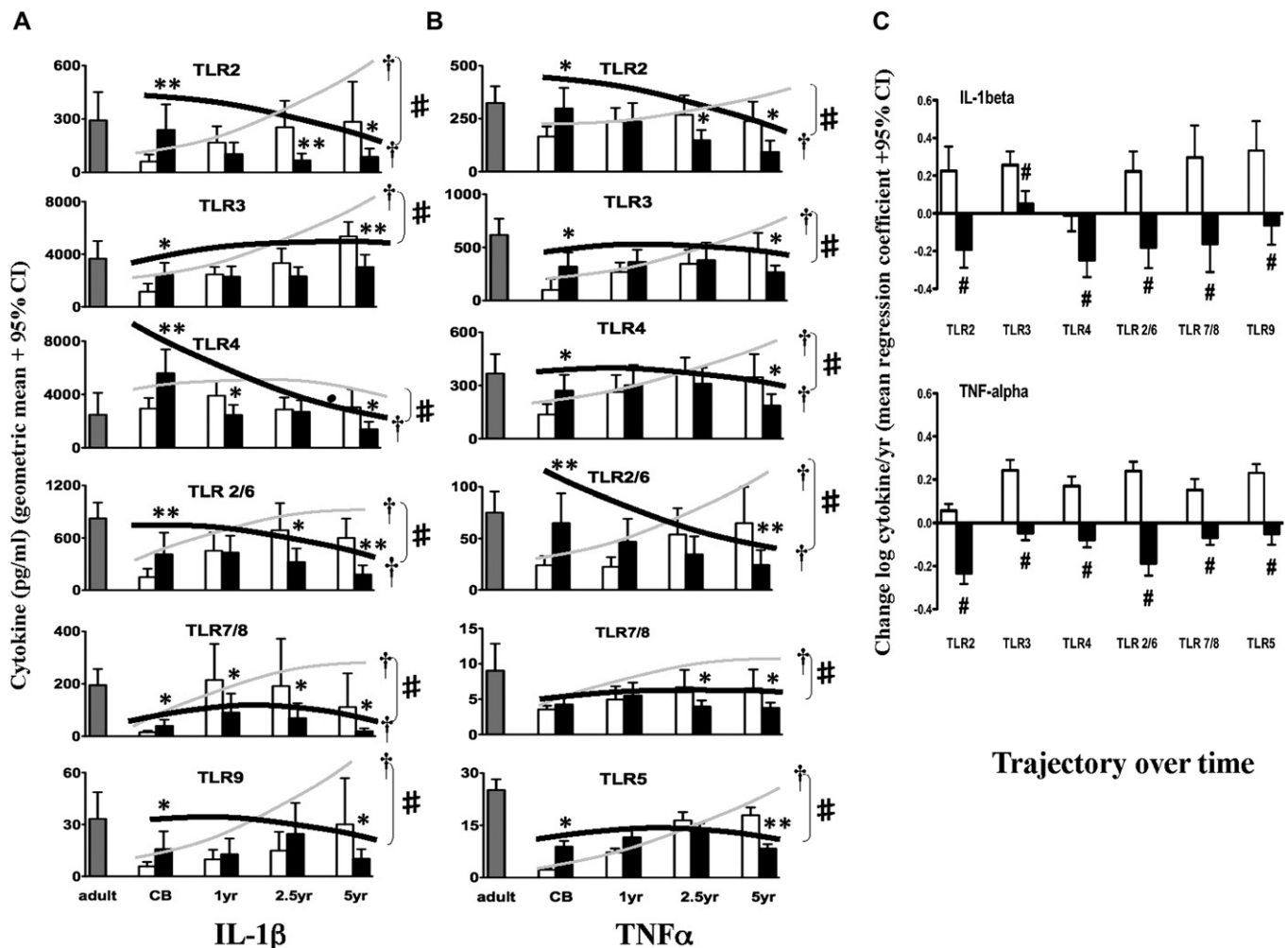


FIG 1. Maturation of innate immune function in allergic and nonallergic children during their first 5 years. **A** and **B**, IL-1 β (Fig 1, A) and TNF- α (Fig 1, B) responses after TLR stimulation in nonallergic (white columns, $n = 35$) and allergic (black columns, $n = 35$) children. Adult responses are shown in shaded columns ($n = 10$). Results are shown as geometric means with 95% CIs. * $P < .05$ and ** $P < .001$ denote between-group and † $P < .05$ denotes within-group differences. **C**, Differences in trajectory (slope) of cytokine responses between groups over 5 years (# $P < .001$). CB, Cord blood.

and the maturation of allergen-specific T_H1 IFN- γ responses (as discussed further below). Consistent with their nonallergic phenotype, the T_H2-related IL-13 responses in these children remained persistently low throughout their first 5 years of life (Fig 2, B).

Differences in ontogeny of innate and adaptive immune function in allergic children

Allergic children show virtually the opposite developmental trajectory of innate immune function as their nonallergic counterparts (Fig 1, C), beginning with hyperresponsiveness at birth but then showing progressive attenuation, with age leading to deficient responses at 5 years compared with those seen in nonallergic children (depicted schematically in Fig 3).

Differences in innate immune function were already evident at birth, when allergic children showed relative hyperresponsiveness to microbial stimulation compared with the nonallergic group (Fig 1 and Tables II and III). This supports our initial

observations³ in an independent cohort. Specifically, in the present study we observed that the allergic group had significantly higher cord IL-1 β responses to TLR2 ($P < .001$), TLR3 ($P < .05$), TLR4 ($P < .001$), TLR2/6 ($P < .001$), TLR7/8 ($P < .05$), and TLR9 ($P < .05$) ligation at birth compared with nonallergic children (Fig 1, A). Similar hyperresponsiveness was seen for TLR-induced TNF- α (Fig 1, B), IL-6 (Table II), and IL-10 (Table III) production at birth. There was no difference in basal (spontaneous) production of cytokines between the 2 groups at birth (data not shown). It is also notable that responses to many TLR ligands in the allergic group were also at similar or greater levels as those of adults (as shown for TLR2 and TLR4 responses in Fig 1).

In the postnatal period the allergic group showed a relative decrease in their innate immune responses relative to the nonallergic group, with significantly lower cytokine responses than the nonallergic subjects by 5 years of age. Specifically, at the end of the follow-up period, allergic subjects had significantly attenuated IL-1 β (Fig 1, A), TNF- α (Fig 1, B), IL-6 (Table II), and IL-12

TABLE II. Comparison of neonatal IL-6 responses to TLR stimulation in allergic (n = 35) and nonallergic (n = 35) children

IL-6 (pg/mL)	CB		1 y		2.5 y		5 y	
	Nonallergic children	Allergic children	Nonallergic children	Allergic children	Nonallergic children	Allergic children	Nonallergic children	Allergic children
TLR2	4,068 (2,766-5,982)	7,623 (6,207-11,159)*	5,435 (3,597-8,212)	2,533 (1,563-4,106)*	7,103 (4,106-12,288)	3,157 (1,874-5,318)*	971 (428-2,206)	1,684 (911-3,114)
TLR3	4,717 (2,352-9,462)	8,818 (9,357-17,845)*	14,394 (11,194-18,509)	14,550 (10,540-20,087)	19,133 (15,142-24,176)	17,388 (13,546-22,320)	27,860 (25,638-30,274)	16,075 (12,548-20,154)*
TLR4	7,581 (4,602-12,490)	16,268 (11,027-24,000)*	16,070 (12,732-20,283)	16,397 (11,705-22,969)	18,019 (14,135-22,972)	12,310 (9,008-16,187)*	26,260 (23,227-29,689)	12,589 (8,222-18,004)*
TLR5	4,816 (3,098-7,486)	13,164 (8,365-20,715)*	17,953 (12,746-25,288)	12,630 (8,781-18,168)	17,391 (14,129-23,229)	7,723 (4,813-12,392)*	8,831 (6,938-11,849)	2,861 (1,191-5,781)*
TLR2/6	5,367 (2,785-10,344)	14,768 (9,682-22,526)*	13,144 (10,171-16,987)	15,912 (10,899-23,232)	16,165 (10,967-23,824)	22,026 (18,201-26,656)	25,330 (21,155-30,328)	24,463 (20,609-29,039)
TLR7/8	1,758 (1,065-2,903)	3,948 (2,861-6,604)*	5,058 (3,028-8,447)	2,941 (1,739-4,972)	2,422 (1,510-3,884)	2,713 (1,601-4,598)	2,241 (1,163-4,319)	1,225 (531-1,829)*
TLR9	137 (90-210)	184 (111-304)	238 (152-372)	185 (123-280)	221 (149-328)	339 (206-558)	157 (86-284)	217 (130-361)

IL-6 (pg/mL)	Regression coefficient (slope)	
	Nonallergic children	Allergic children
TLR2	-0.19 (-0.39 to -0.004)†	-0.24 (-0.37 to -0.12)†
TLR3	0.33 (0.23 to 0.43)†	0.20 (0.09 to 0.31)††
TLR4	0.22 (0.14 to 0.29)†	0.08 (0.005 to 0.16)††
TLR5	-0.04 (-0.14 to 0.06)	-0.23 (-0.41 to -0.05)††
TLR2/6	0.27 (0.16 to 0.39)†	0.17 (0.07 to 0.26)††
TLR7/8	-0.03 (-0.20 to 0.13)	-0.14 (-0.33 to 0.05)
TLR9	-0.02 (-0.14 to 0.09)	0.05 (-0.06 to 0.15)

Results are shown as geometric means with 95% CIs.

CB, Cord blood.

* $P < .05$ versus the nonallergic group at the same age.† $P < .05$ denotes a significant change in cytokine responses over time within each group.‡ $P < .05$ denotes a significant difference in the trajectory of cytokine responses over time between the 2 groups where slope is the change in log cytokine per year and shown as means with 95% CIs.

(see Table E1) responses to most TLR ligands compared with nonallergic children.

The age of transition from relative hyperresponsiveness to relative deficient responses in the allergic group varied according to the cytokine produced. For example, this occurred within the first year of life for IL-1 β (Fig 1, A) and by 2.5 years for TNF- α (Fig 1, B) and IL-6 (Table II). Hyperresponsiveness in IL-12 responses was seen at 1 year of age in the allergic group but was significantly deficient by 5 years of age (see Table E1). For IL-10, persistent hyperresponsiveness was still seen to most TLR ligands at 2.5 years but not by the age of 5 years (Table III). The tendency for increased (in nonallergic children) or decreased (in allergic children) responses over time within the group is likely to reflect differences in the kinetics of postnatal cytokine maturation between individuals. The age-related differences between groups are shown in Fig 1, C, and represented schematically on Fig 3.

As anticipated, the allergic group also showed significant differences in development of their postnatal adaptive T-cell responses. Consistent with our first studies,² allergic children had attenuated development of T_H1 function relative to nonallergic subjects, with significantly lower IFN- γ responses to allergens and mitogens over the first 5 years of life (Fig 2, A). They also had significantly higher IL-13 responses to these stimuli throughout the first 5 years of life compared with nonallergic subjects (Fig 2, B). Notably, in this population the higher allergen-specific IL-13 responses were already evident at birth and correlated

with the postnatal IL-13 responses ($r = 0.33$ and $P = .007$ at 1 year), which increased with age in allergic subjects. There were no significant differences in IL-10 responses to allergens or PHA mitogen between the groups (data not shown).

Association between early innate immune responses and T-cell development

First, we examined the relationship between innate immune development and T_H1 immune ontogeny, correlating the change of each of these measures with age. As anticipated, the nonallergic children showed very strong and highly significant positive correlations between the development of innate responses (particularly TLR4- and TLR9-mediated response) and T_H1 maturation. For example, allergen-specific IFN- γ correlated with TLR4-induced ($r = 0.94$, $P = .005$) and TLR9-induced ($r = 0.89$, $P = .007$) TNF- α production, and mitogen-induced IFN- γ correlated with TLR4-induced ($r = 0.94$, $P = .005$) and TLR9-induced ($r = 0.89$, $P = .007$) TNF- α . This was not seen in the allergic group, which did not show any consistent correlations (data not shown). This suggests a close relationships between the innate immune development and T-cell maturation in nonallergic, but not allergic, children.

We then examined the relationship between early innate immune responses and the predisposition for T_H2 differentiation in allergic subjects. A greater propensity for innate inflammatory

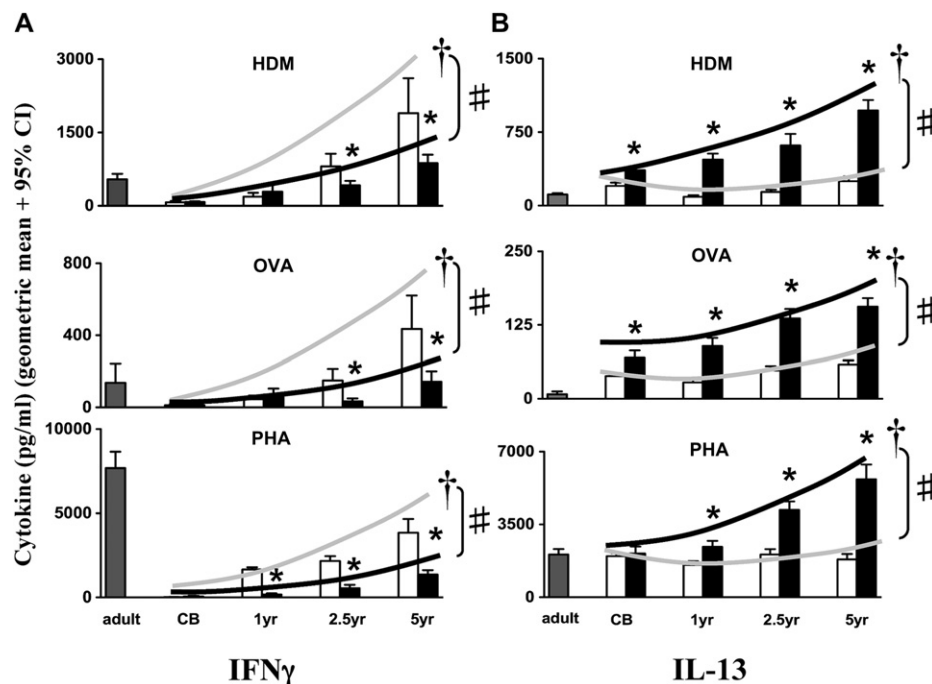
TABLE III. Comparison of neonatal IL-10 responses to TLR stimulation in allergic (n = 35) and nonallergic (n = 35) children

IL-10 (pg/mL)	CB		1 y		2.5 y		5 y	
	Nonallergic children	Allergic children	Nonallergic children	Allergic children	Nonallergic children	Allergic children	Nonallergic children	Allergic children
TLR2	301 (231-391)	537 (382-755)*	389 (310-488)	660 (504-865)*	531 (364-775)	948 (644-1,393)*	204 (110-378)	513 (303-869)*
TLR3	491 (372-649)	931 (629-1,378)*	640 (445-920)	963 (678-1,367)	379 (288-498)	496 (347-708)	668 (451-989)	262 (157-439)*
TLR4	299 (165-541)	795 (542-1,167)*	434 (318-593)	877 (587-1,311)*	638 (413-986)	1,069 (1,041-1,543)*	454 (334-616)	668 (447-998)
TLR5	11 (8-14)	24 (18-32)*	19 (14-26)	36 (27-48)*	46 (35-60)	77 (62-96)*	63 (49-80)	38 (28-52)*
TLR2/6	26 (20-33)	56 (38-82)*	48 (35-65)	91 (66-126)*	60 (43-84)	126 (85-187)*	98 (68-141)	41 (28-61)*
TLR7/8	125 (85-183)	219 (160-301)*	170 (136-212)	291 (211-399)*	156 (114-215)	320 (234-437)*	229 (164-318)	108 (68-172)*
TLR9	254 (197-328)	295 (219-397)	193 (150-249)	244 (192-311)	250 (166-375)	266 (200-353)	251 (188-334)	321 (240-429)

IL-10 (pg/mL)	Regression coefficient (slope)	
	Nonallergic children	Allergic children
TLR2	-0.06 (-0.20 to 0.07)	-0.03 (-0.15 to 0.08)
TLR3	0.03 (-0.05 to 0.10)	-0.25 (-0.37 to -0.13)†‡
TLR4	0.07 (-0.01 to 0.16)	-0.04 (-0.13 to 0.05)‡
TLR5	0.35 (0.28 to 0.42)†	0.09 (0.02 to 0.17)†‡
TLR2/6	0.23 (0.15 to 0.32)†	-0.03 (-0.12 to 0.05)‡
TLR7/8	0.09 (-0.02 to 0.20)	-0.11 (-0.21 to -0.005)†‡
TLR9	-0.04 (-0.13 to 0.06)	0.01 (-0.08 to 0.10)

Results are shown as geometric means with 95% CIs.

CB, Cord blood.

P* < .05 versus the nonallergic group at the same age.†*P* < .05 denotes a significant change in cytokine responses over time within each group.‡*P* < .05 denotes a significant difference in the trajectory of cytokine responses over time between the 2 groups where slope is the change in log cytokine per year and shown as means with 95% CIs.FIG 2.** Maturation of adaptive T cell immune function in allergic and nonallergic children during their first 5 years. **A** and **B**, IFN- γ (**Fig 2, A**) and IL-13 (**Fig 2, B**) responses to house dust mite (HDM), ovalbumin (OVA), and PHA in nonallergic (white columns, n = 35) and allergic (black columns, n = 35) children. Adult responses are shown in shaded columns (n = 10). Results are shown as geometric means with 95% CIs. **P* < .05 denotes between-group and †*P* < .05 denotes within-group differences. #*P* < .05 denotes differences in the trajectory of cytokine responses between groups over 5 years. CB, Cord blood.

responses in the perinatal period was significantly correlated with the propensity for allergen-specific T_H2 responses in the early postnatal period (Table IV). Specifically, there were consistent

positive correlations between the level of inflammatory cytokine responses at birth and developing IL-13 responses at birth and during the first year of life (Table IV). This was particularly

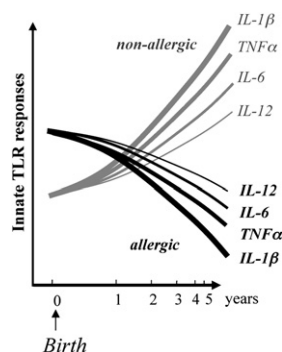


FIG 3. Trajectory of innate immune development of allergic and nonallergic children: schematic representation of the trajectory of innate TLR responses with age in nonallergic (gray lines) and allergic (black lines) children.

true for IL-1 β but also for TNF- α and IL-6. It is notable that the same trends were observed for virtually all of these repeated independent measures of innate immune function (examining different cytokines in response to different TLR ligands), although not all were statistically significant. It has been validly argued^{13,14} that consistent and repeated relationships such as these reinforce likely and biologically relevant interactions (despite the fact that some of these significant relationship would be abolished by statistical corrections for multiple comparisons). We propose that these repeated observations collectively support a plausible relationship that needs to be explored further. These findings are also consistent with our hypothesis that an increased propensity for inflammatory cytokine production by APCs in the perinatal period might be implicated in T_H2 differentiation in the developing T-cell compartment.

Ontogeny of TLR expression on APCs from allergic and nonallergic children

In our previous studies we identified that higher TLR2-mediated cytokine responses are associated with increased TLR2 expression on pDCs.² In the present study we investigated this further as a possible mechanism of the increased innate responses observed in the allergic group. Specifically, we examined the ontogeny of TLR expression on monocytes, NK cells, mDCs, and pDCs in a randomly chosen subgroup of allergic and nonallergic children. The main finding was significantly increased TLR2 expression per pDC in the blood of allergic subjects at birth ($P < .001$) and 1 year of age ($P < .01$), notably during the same period when they are displaying hyperresponsiveness relative to the nonallergic group (Fig 4). This contrasted with the nonallergic children, who showed relatively unchanged expression over the first 5 years. There were no other differences found in maturation of TLR expression on monocytes, NK cells, or mDCs between the 2 groups of children (data not shown).

DISCUSSION

Interactions between the microbial environment and the developing immune system are of critical interest in understanding the modern epidemic of immune disease, which has occurred in a context of decreasing microbial burden.¹ Differences in genetic propensity and early environmental interactions appear to contribute to differences in disease predisposition and variations in phenotype. Here, for the first time, we map the longitudinal development of innate microbial recognition pathways in nonallergic

TABLE IV. Relationship between TLR-mediated innate immune responses (IL-1 β , TNF- α , and IL-6) at birth and early allergen-specific T_H2 cytokine (IL-13) responses

	IL-13 responses at birth		IL-13 responses at 1 y	
	<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
IL-1 β at birth				
TLR2	0.29	.02*	0.29	.02*
TLR3	0.32	.01*	0.32	.09
TLR4	0.32	.01*	0.32	.04*
TLR2/6	0.29	.02*	0.13	.05*
TLR7/8	0.19	.15	0.19	.02*
TLR9	0.28	.02*	0.28	.01*
TNF- α at birth				
TLR2	0.64	.06	0.27	.03*
TLR3	0.22	.10	0.23	.08
TLR4	0.21	.11	0.22	.06
TLR2/6	0.64	.06	0.34	.01*
TLR7/8	0.15	.24	0.67	.05*
TLR5	0.37	.02*	0.58	.01*
IL-6 at birth				
TLR2	0.27	.03*	0.13	.28
TLR3	0.16	.22	0.51	.08
TLR4	0.16	.22	0.48	.09
TLR5	0.10	.42	0.25	.04*
TLR7/8	0.16	.21	0.17	.17
TLR9	0.23	.06	0.52	.08

Statistically significant relationships ($P < .10$ and $*P < .05$) are shown in boldface.

children and show striking developmental differences in children with allergic disease.

After relatively reduced TLR activity at birth, healthy nonallergic children show an age-related increase in the responsiveness of these microbial recognition pathways. Nonallergic children also have a corresponding age-related maturation in their adaptive T-cell compartment with T_H1 consolidation, as noted in our original studies.² These observations provide new evidence that T-cell development and in particular reduced T_H2 differentiation might be driven through the innate immune system as it undergoes microbe-driven maturation. These relationships are distorted in allergic children, with altered developmental trajectory of both innate and adaptive immunity, as well as apparent “dissociation” between these functional cellular compartments of the immune system.

The first consistent difference in TLR-mediated microbial responses is already evident at birth, with significantly increased inflammatory cytokine production to a wide range of TLR ligands in the allergic group. IL-10 responses are also significantly increased compared with those in the nonallergic group, reproducing our previous observations³ in an independent group of children. This strongly suggests that *in utero* gene-environment interactions lead to variations in innate immune function that might modify postnatal immune development and disease predisposition.

Although innate immune responses play an important role in host defense in the perinatal period before T-cell function is established, excessive inflammatory responses are maladaptive and can lead to unwanted tissue damage. Lower responses in the nonallergic children suggest that these “measured” responses might be more conducive to normal adaptive postnatal immune maturation. Indeed, our findings confirm very early presymptomatic emergence of T_H2-dominant allergen-specific responses in the allergic group, which is already evident at birth and increasing

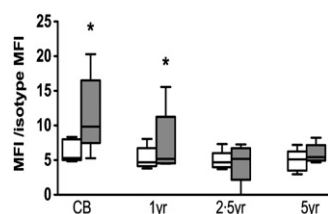


FIG 4. TLR2 expression on pDCs in allergic and nonallergic children during their first 5 years. TLR2 expression on circulating pDCs in nonallergic (open columns, $n = 5$) and allergic (gray columns, $n = 5$) children at birth and 1, 2.5, and 5 years of age is shown. Results are expressed as the mean fluorescence intensity (MFI) of TLR2⁺ pDCs above isotype control and shown as box-and-whisker plots with the 5th and 95th percentiles. The line inside the box represents the median. * $P < .05$ versus age-matched nonallergic control subjects. CB, Cord blood.

with age. We speculate that the increased propensity to produce innate inflammatory cytokines (IL-1 β , TNF- α , and IL-6 or increased innate immune activity) is implicated in this T_H2 proclivity, potentially “tipping the balance” during this critical period of T-cell development. Recent evidence in mice showing low maternal inflammatory response to *Acinetobacter lwoffii* F78 bacterium to be associated with reduced neonatal innate immune responses and reduction in their risk of allergic disease in later life¹⁵ support our findings. In addition to their recognized T_H2 properties, IL-4 and IL-13 also have well-recognized potent anti-inflammatory properties that are important in downregulating LPS-mediated proinflammatory cytokine production in human monocytes.¹⁶ It is possible that higher production of T_H2 cytokines in allergic children is initiated as a part of the negative feedback mechanism to dampen the higher innate inflammatory responses. Proinflammatory cytokines can also play a crucial role in altering the balance between tolerance and inflammation through regulatory pathways. Dominitzki et al¹⁷ have shown that IL-6 abrogates the *de novo* induction of adaptive regulatory T cells by interfering with conversion of naive T cells into regulatory T cells. These observations highlight the need to investigate inflammatory pathways further in the pathogenesis of the allergic response. It is also notable that despite these exaggerated early innate responses in the allergic group, T_H1 immune maturation remained attenuated relative to the nonallergic children, suggesting a dissociation between the innate and adaptive immune compartments in allergic children, which also needs to be investigated further.

The second major difference in allergic children was that although the early innate hyperresponsiveness was sustained for a variable period (depending on the ligand and cytokine), this ultimately progressed to a relatively attenuated response to the same microbial products. By 5 years of age, production of most cytokines was significantly lower than in the nonallergic subjects. This is consistent with a recent cross-sectional comparison of TLR2 responses in 5-year-old children, which found that monocytes from allergic children had impaired regulation of TLR2 on peptidoglycan stimulation, suggesting a relatively hyporesponsive state.¹⁸

Microbial exposure is critical for promoting normal immune development,¹⁹ and we have previously proposed that this might be even more critical to overcome allergic propensity in predisposed subjects.² The relatively clean environmental setting in the present study could therefore be contributing to the observed differences in innate immune development. We propose that in a more traditional environment, where allergic disease is uncommon, higher microbial exposure might help normalize immune

development in these otherwise predisposed children, so that they more closely approximate nonallergic children. If this were the case, our current findings would suggest that the effects of microbial burden on innate responses depend on age; that is, maternal microbial exposure might serve to dampen the perinatal propensity for innate hyperresponsiveness seen in the allergic children to prevent inflammation, as argued by Conrad et al.¹⁵ Conversely, subsequent postnatal microbial exposure serves to promote measured postnatal maturation to achieve a normal non-allergic profile. This is consistent with other evidence that the effects of postnatal microbial exposure vary according to host age and maturity.^{20,21}

Finally, differences in TLR receptor expression in allergic infants might provide one possible mechanism for differences in innate function. We have shown that allergic children have more TLR2⁺ pDCs at birth, and this increase is maintained for at least the first year of life. TLR ligand-activated dendritic cells are known to produce high levels of IL-6,²² and we previously detected significant correlations between TLR2 expression on pDCs and TLR2-induced cytokine expression.³ At this stage, the significance of this is unclear, particularly because the differences in function of other TLR pathways in that study were not associated with variations in receptor expression.

In summary, this study provides an essential foundation for understanding normal immune development. Clear age-related differences in microbial responses of allergic children provide new insights into the pathogenesis of disease, suggesting a role of innate inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , in the early propagation of the allergic response. These findings highlight the need to further investigate disordered interaction between the innate and adaptive arms of the immune response in the evolution of the allergic phenotype. Consistent perinatal differences in innate immune function further accentuate the likely importance of antenatal determinants of both neonatal immune function and the developmental trajectory in the postnatal period.

We thank all the children and parents, as well as the research staff, obstetricians, and midwives, involved in this longitudinal study. We also thank Dr Brad Zhang and Dr Nick de Clerk for their expert statistical advice.

Key messages

- This is the first longitudinal study to document the normal development of TLR-mediated microbial responses over the first 5 years of life and to show dramatic differences in allergic children.
- Our observations directly challenge notions that allergic disease is preceded by reduced capacity for innate immune responses but rather suggests that dysregulated inflammatory responses might have a role in abnormal T-cell development.
- This study provides fundamental information that is an essential prelude to any future immunomodulatory therapies using microbial products.

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TABLE E1. Comparison of neonatal IL-12 responses to TLR stimulation in allergic (n=35) and non-allergic (n=35) children

IL-12 (pg/mL)	CB		1 y		2.5 y		5 y	
	Nonallergic children	Allergic children	Nonallergic children	Allergic children	Nonallergic children	Allergic children	Nonallergic children	Allergic children
TLR4	297 (204-431)	221 (143-340)	428 (306-599)	857 (556-1,321)*	1,350 (960-1,897)	774 (568-1,054)	553 (344-890)	288 (208-398)*
IL-12 (pg/mL)	Regression coefficient (slope)							
	Nonallergic				Allergic			
TLR4	0.14 (0.04 to 0.23)				0.00 (−0.10 to 0.10) ^{†‡}			

Results are shown as geometric means with 95% CIs.

CB, Cord blood.

* $P < .05$ versus the nonallergic group at the same age.

[†] $P < .05$ denotes a significant change in cytokine responses over time within each group.

[‡] $P < .05$ denotes a significant difference in the trajectory of cytokine responses over time between the 2 groups where slope is the change in log cytokine per year and shown as means with 95% CIs.