

Patterns of immune development in urban preschoolers with recurrent wheeze and/or atopy

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Background: Disadvantaged urban children have high rates of allergic diseases and wheezing, which are diseases associated with type 2-biased immunity.

Objective: We sought to determine whether environmental exposures in early life influence cytokine responses that affect the development of recurrent wheezing illnesses and allergic sensitization.

Methods: A birth cohort of 560 urban families was recruited from neighborhoods with high rates of poverty, and 467 (83%) children were followed until 3 years of age. Cytokine responses were measured in blood cell samples obtained at birth (cord blood) and ages 1 and 3 years. Cytokine responses were examined in relation to personal characteristics and environmental exposures to allergens and endotoxin and to the development of allergic sensitization and recurrent wheeze assessed at age 3 years.

Results: Cytokine responses generally increased with age, but responses at birth were poorly predictive for those at ages 1 and 3 years. Exposure to certain allergens (cockroach, mouse, dust mite) was significantly associated with enhanced cytokine responses at age 3 years, including IFN- α and IL-10 responses to certain stimulants and responses to phytohemagglutinin. Regarding the clinical outcomes, reduced LPS-induced IL-10 responses at birth were associated with recurrent wheeze. In contrast, reduced respiratory syncytial virus-induced IL-8 responses and increased 5'-cytosine-phosphate-guanine-3' (CpG)-induced IL-12p40 and allergen-induced IL-4 responses were associated with atopy.

Conclusions: These findings suggest that diverse biologic exposures, including allergens and endotoxin, in urban homes stimulate the development of cytokine responses in early life, and that cytokine responses to specific microbial and viral stimuli are associated with the development of allergic sensitization and recurrent wheeze. (*J Allergy Clin Immunol* 2017;■■■:■■■-■■■.)

Key words: Cytokines, cord blood, mononuclear cells, immune development, wheezing, allergy, children

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Wheezing illnesses and atopy during infancy are the 2 main risk factors for persistent childhood asthma, and understanding how immune development relates to the onset of these conditions is an important step toward asthma prevention.¹ Patterns of immunologic development in early life, such as low interferon responses, blunted responses to innate stimuli, and a bias toward T_H2-like cytokine responses, indicate increased risk for developing both wheezing illnesses and allergic sensitization.² Overall, it appears that children with a sluggish rate of immune maturation or an imbalance in the development of effector to regulatory mechanisms are at increased risk for both allergic sensitization and increased severity of viral respiratory illnesses.³ There is a growing appreciation that early life exposures to microbial stimuli and innate immune responses to these stimuli may guide differentiation of both effector and regulatory T cells and thereby influence the development of allergic sensitization. Children who grow up in urban environments have especially high rates

Abbreviations used

CpG: 5'—cytosine—phosphate—guanine—3'
 CR: Cockroach extract
 DM: Dust mite extract
 LASSO: Least absolute shrinkage and selection operator
 LPS: Lipopolysaccharide
 PBMC: Peripheral blood mononuclear cells
 PHA: Phytohemagglutinin
 RSV: Respiratory syncytial virus
 TT: Tetanus toxoid
 URECA: Urban Environment and Childhood Asthma

of allergies and respiratory illnesses,^{4,5} but how specific aspects of the urban environment influence immune development and subsequent clinical outcomes are incompletely understood.

The Urban Environment and Childhood Asthma (URECA) birth cohort study was initiated in 2004 to address this knowledge gap.^{6,7} In the URECA study, cord blood mononuclear cells and peripheral blood mononuclear cells (PBMCs) at ages 1 and 3 were stimulated and tested for cytokine responses in an effort to relate early immune development to recurrent wheezing and allergic sensitization. The current analysis of data from birth to age 3 years focuses on 3 main questions. First, what are the patterns of immune development in the first 3 years in urban children at high risk for wheezing and atopy? Second, what are the personal and environmental factors that influence immune development? Finally, what are the patterns of immune development that are associated with wheezing illnesses and atopy? The study tested several hypotheses related to these questions: (1) personal characteristics such as ethnicity, birthweight, season of birth, and sex affect immune development; (2) urban environmental exposures such as high-level cockroach exposure can differentially influence immune responses to promote type 2 cytokine responses; (3) low innate immune responses, particularly interferon responses, are a risk factor for recurrent wheeze and allergic sensitization; and (4) allergic sensitization is related to progressive bias toward type 2 cytokine responses in response to stimulation with both allergenic proteins (cockroach, dust mite) and antigens (eg, tetanus toxoid [TT]).

METHODS**Study design**

URECA is an observational birth cohort study in 4 urban areas of the United States—Baltimore, Boston, New York City, and St Louis, and a main study goal is to identify environmental exposures that affect immune development and the onset of atopy, recurrent wheeze, and ultimately asthma.⁷ Selection criteria included residence in an area with >20% residents below the poverty level, mother or father with allergic rhinitis, eczema, and/or asthma, ≥34 weeks gestation, and collection of a cord blood sample. A maternal questionnaire was administered prenatally. At delivery, cord blood and information from the hospital record were collected. During the first 3 years of life, questionnaires were administered every 3 months either by phone or in person. The child was seen at the research clinic at 12, 24, 33, and 36 months. Between February 2005 and March 2007, 1850 families were screened, 889 met eligibility criteria, and 560 were enrolled.

Study assessments

Total and allergen-specific IgE. Annually specific IgE levels (ImmunoCAP; Phadia, Uppsala, Sweden) were measured for German cockroach. In addition, at 2 and 3 years, specific IgE for dust mite, dog, cat,

mouse, and *Alternaria* were measured. Prick skin testing (Multi-Test II, Lincoln Diagnostics, Decatur, Ill) was performed at age 33 to 36 months for 14 indoor and outdoor allergens (Greer Laboratories, Lenoir, NC): American and German cockroach mix, German cockroach, cat, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, dog, mouse, rat, *Alternaria*, *Aspergillus*, *Penicillium*, and ragweed, mixed grass, and mixed tree pollens. Wheal sizes ≥3 mm larger than the saline control or sIgE ≥ 0.35 kU/L were considered positive.

Home environmental assessments. Three home visits occurred: at 3 months, between 1 and 2 years of age, and between 2 and 3 years of age. URECA staff visited the child's home to do an environmental survey and to collect household dust samples, which were assayed for Bla g 1 (German cockroach), Can f 1 (dog), Fel d 1 (cat), Der f 1 (*Dermatophagoides farinae*), Der p 1 (*Dermatophagoides pteronyssinus*), and Mus m 1 (mouse) by 2-site monoclonal antibody ELISAs (Indoor Biotechnologies, Inc, Charlottesville, Va). First- and third-year samples were also analyzed for endotoxin by the recombinant factor C assay.⁸

Cytokine responses. Blood mononuclear cells freshly isolated from cord, age 1 year, and age 3 years samples were incubated with a panel of innate stimuli (including viruses), polyclonal stimuli, and antigens (see Table E1 in this article's Online Repository at www.jacionline.org). These stimuli included LPS, polyinosinic-polycytidylic acid, peptidoglycan, 5'—cytosine—phosphate—guanine—3'-A (CpG)-A, respiratory syncytial virus (RSV), rhinovirus, PHA, cockroach extract (CR), dust mite extract (DM), TT, and mAb specific for CD3 and CD28. Culture supernatants were tested for cytokine responses using a bead-based multiplex assay (Beadlyte; Upstate Biotechnology, Lake Placid, NY; or Milliplex; EMD Millipore, Billerica, Mass). Cytokines were selected based on involvement with specific innate and adaptive immune responses.⁹ Laboratory equipment and procedures for the 4 site laboratories were carefully standardized, and reagents were centrally purchased in large batches and supplied to each site. Quality control tests were conducted approximately yearly to determine reproducibility of the same sample processed in different laboratories, as previously described.⁹

Definitions

Recurrent wheeze was defined as at least 2 lifetime wheezing episodes, with at least 1 episode occurring in the third year. Eczema was defined as a score ≥1.0 on the Eczema Area and Severity Index on at least 1 scheduled research visit.⁷ Atopy was defined as at least 1 positive skin test or detectable serum aeroallergen-specific IgE at age 3 years.

Statistical analysis

Cytokine, dust allergen, and endotoxin measurements were log-transformed for all analyses. Cytokine and allergen values that were below the lower limit of detection were imputed using Tobit regression. In addition, missing cytokine response values were imputed using R's (R Foundation, Vienna, Austria) package MICE (Multivariate Imputation by Chain Equations) via the random forest algorithm. The percentage of missing data is as follows: innate panel responses 20.8%, adaptive panel cytokines 20.6%, and dust allergen levels 19.4%. Altogether, 100 training datasets and 50 test datasets were imputed. The training datasets were used to create the models, and the test datasets were used to develop the final estimates, by averaging the results according to the rules of multiple imputation.¹⁰

Associations among the cytokines at every year, and associations of cytokine responses with population characteristics and exposure data were estimated using Fisher Z-transformed Pearson correlations. Univariate (ie, crude) odds ratios between cytokine responses and outcomes were estimated using logistic regression. Departure from linearity for the cytokine effect was also examined by testing for the significance of a cubic spline term in the logistic regression. To account for multiple comparisons in analyses among cytokines, allergen exposures, and clinical outcomes, an adjusted *P* value controlling for false discovery rate was calculated.

To examine the relationships of cytokine responses to recurrent wheeze and atopy in 1 multivariate model, Least Absolute Shrinkage and Selection Operator (LASSO) regression was applied to the 100 training datasets. The

LASSO and logistic regressions, adjusted for child's ethnicity and sex, were performed separately for each year (birth, year 1, and year 3) and also using all cytokine responses across all the years. Additional details regarding LASSO are provided in this article's Online Repository (at www.jacionline.org).

RESULTS

Study population

The cohort consisted of 560 inner-city children of whom 467 (83%) had sufficient data in the third year to characterize recurrent wheeze (Table I). Children were predominantly ethnic minorities, two-thirds of participating families were single mothers, and median household income was <\$15,000 per year (Table I). There were no significant differences between those families who were included versus those excluded from the analysis population (see Table E2 in this article's Online Repository at www.jacionline.org). At age 3 years, 36% had recurrent wheeze, 44% were sensitized to at least 1 aeroallergen, and 9% had a physician diagnosis of eczema.¹¹ Children with recurrent wheeze had a median of 6 wheezing episodes (range 2-26) by age 3 years and were more likely to have been prescribed albuterol (77% vs 22%), an inhaled corticosteroid (27% vs 5%), or an oral corticosteroid (33% vs 7%) than were children without recurrent wheeze.

Patterns of immune development

Most cytokine responses increased with age, and this was particularly evident for antigen-induced responses. Tetanus-induced responses had a large increase between birth and age 1 year and thereafter were stable or slightly decreased (see Fig E1 in this article's Online Repository at www.jacionline.org). In contrast, allergen-specific responses were generally greatest at age 3 years. Effects of age were more variable for responses to innate stimuli and viruses. Notably, IL-12p40 responses were generally greatest at birth (see Fig E2 in this article's Online Repository at www.jacionline.org).

A network analysis to test for relatedness of cytokine responses at birth to predict subsequent patterns of immune development demonstrated a number of significant associations that were predominantly found within a particular age group (Fig 1). There were no significant associations between responses at birth and those at subsequent years. However, there were a few associations between age 1 year and age 3 years in IL-4, IL-5, and IL-13 responses to TT and PHA. These findings suggest that cytokine responses at birth have a limited capacity to predict subsequent cytokine responses.

Personal factors and cytokine responses

We previously reported that birth weight/gestational age, ethnicity, and season of birth were significantly associated with cord blood cytokine responses¹²; the latter 2 factors were also associated with several cytokine responses at age 3 years (see Figs E3 and E4 in this article's Online Repository at www.jacionline.org). For example, African American children compared with other participants (mostly Hispanic) had greater responses to viruses and greater type 2 responses to polyclonal stimuli. Furthermore, season of birth affected type 2 cytokine responses to TT, IL-8 responses to rhinovirus and poly IC, and IL-10 responses to a variety of stimuli; these responses were greatest for children born during winter (January-March) (Fig 2). Sex had

TABLE I. Demographic characteristics of the analysis population

	Analysis population n = 467
Study site	
Baltimore	140 (30.0)
Boston	108 (23.1)
New York	93 (19.9)
St Louis	126 (27.0)
Mother smoked during pregnancy	83 (17.8)
No. of smokers in the home	
0	235 (50.5)
1-2	203 (43.7)
3+	27 (5.8)
Mother has high school education	273 (58.6)
Mother is married	64 (13.7)
Mother has ever had asthma	234 (50.3)
Age of mother at time of birth (y)	24.5 ± 5.9
Household income <\$15,000	321 (68.7)
Child's ethnicity	
African American	333 (71.3)
Hispanic	92 (19.7)
Other/mixed	42 (9.0)
Child's sex: male	240 (51.4)
Caesarian delivery	154 (33.0)
Gestational age of child at delivery (wks), mean ± SD	38.8 ± 1.5
Child's birth weight (g), mean ± SD	3258 ± 504
Season child was born in	
January-March	113 (24.2)
April-June	117 (25.1)
July-September	138 (29.6)
October-December	99 (21.2)
Ever breastfed child	255 (56.0)
Breastfed child in first 3 months	100 (23.9)

Values are n (%) unless otherwise indicated.

little effect on developmental patterns of cytokine responses (data not shown).

Effects of allergen exposure on cytokine responses

We tested whether exposure to cockroach and dust mite allergens in house dust was associated with specific patterns of allergen-specific cytokine responses at 1 and 3 years of age. Cockroach exposure was not significantly associated with cockroach-specific PBMC responses, whereas dust mite exposure in years 2 and 3 was associated with increased DM-specific IL-10 responses. Contrary to our original hypothesis, neither exposure to CR nor DM proteins in early life promoted allergen-specific T_H2 responses.

There were also significant associations between exposure to allergens and cytokine responses that were not allergen-specific. CR exposure during years 1 to 3 was associated with enhanced PHA-induced cytokine responses. In addition to a positive association with DM-induced IL-10, DM exposure in year 3 was also significantly and positively associated with IL-10 responses to several innate and adaptive stimuli (Fig 3, A and B).

We previously reported that early exposure to CR, mouse, and cat proteins was associated with a reduced risk for recurrent wheezing episodes in the URECA population,¹¹ suggesting that these exposures might affect immune development to enhance antiviral responses. In fact, CR exposure in year 3 was positively

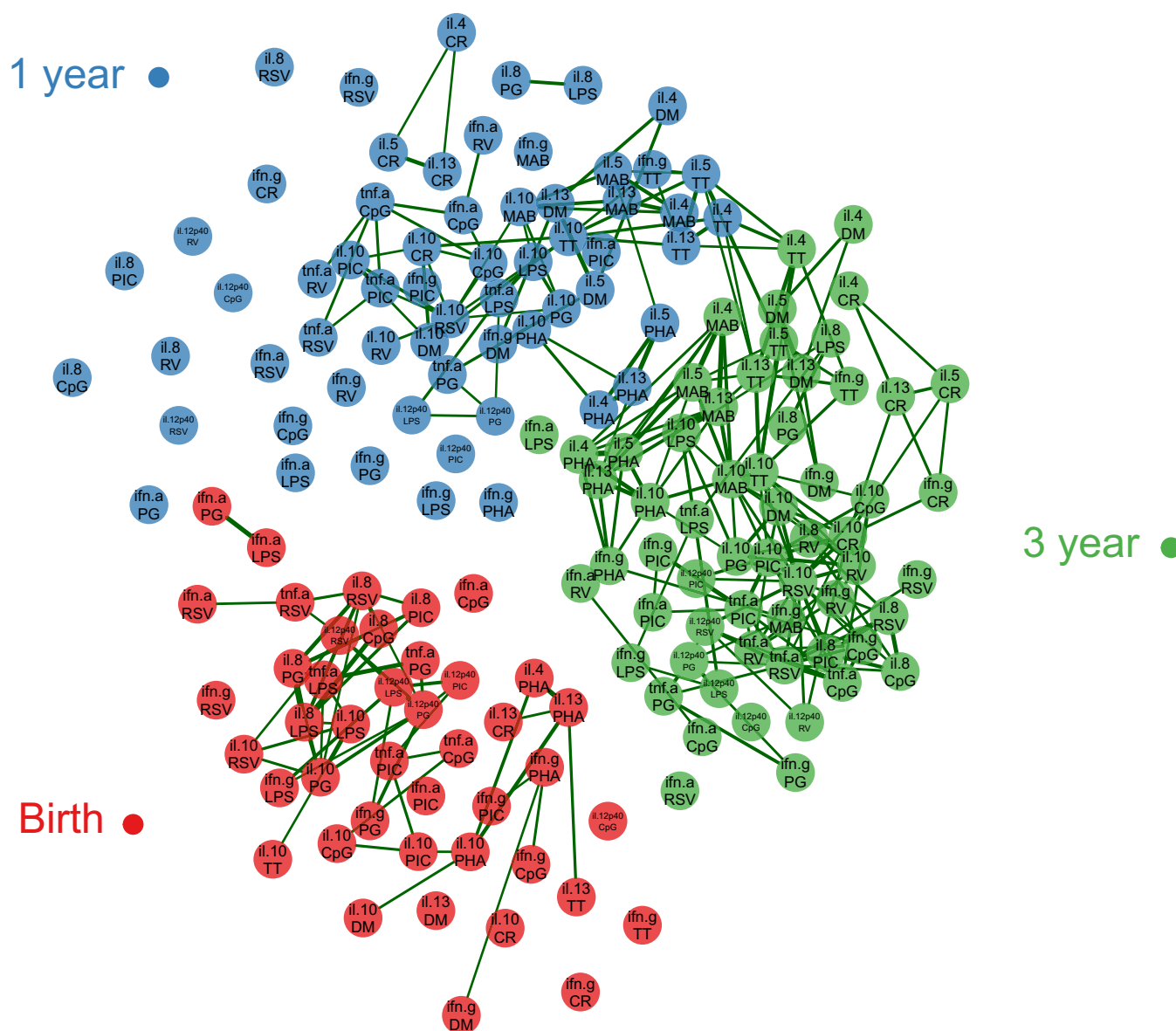


FIG 1. Network analysis of cytokine by stimulant data measured longitudinally. The map uses the correlation matrix among the 164 variables as the proximity matrix to create the network. More highly correlated variables are closer to each other. Correlations > 0.26 (95th percentile) in absolute value are represented with lines. The age at which the cytokine responses were assessed is color-coded. *PIC*, Polyinosinic-polycytidylic acid; *PG*, peptidoglycan; *RV*, rhinovirus.

associated with rhinovirus-induced IFN- α , and there was a similar trend in year 2. Mouse exposure in year 3 was significantly associated with IFN- α responses to LPS, but not respiratory viruses. Cat exposure was not significantly related to antiviral responses.

A few significant associations were noted for other exposures after controlling for multiple comparisons. For example, endotoxin exposure in year 1 was associated with enhanced PHA-induced IL-4 and IL-13 responses, and endotoxin exposure in year 3 had similar trends (Fig 3, B). Exposure to cat in year 2 was inversely associated with PHA-induced IFN- γ and IL-4, but this was not a consistent finding for exposure in other years.

Associations between cytokine responses, recurrent wheeze, and atopy

We next tested 2 hypotheses relating immune development to recurrent wheezing and atopy: (1) low innate immune responses, particularly interferon responses, are a risk factor for recurrent wheeze and allergic sensitization; and (2) allergic sensitization is related to progressive bias toward type 2 cytokine responses to allergens (cockroach, dust mite) and antigens (TT). In the univariate model of innate responses at birth (Fig 4), low LPS-induced IL-10 secretion, but not IFN- α or IFN- γ responses, were related to recurrent wheeze. In fact, recurrent wheeze was positively associated with several responses to innate stimuli,

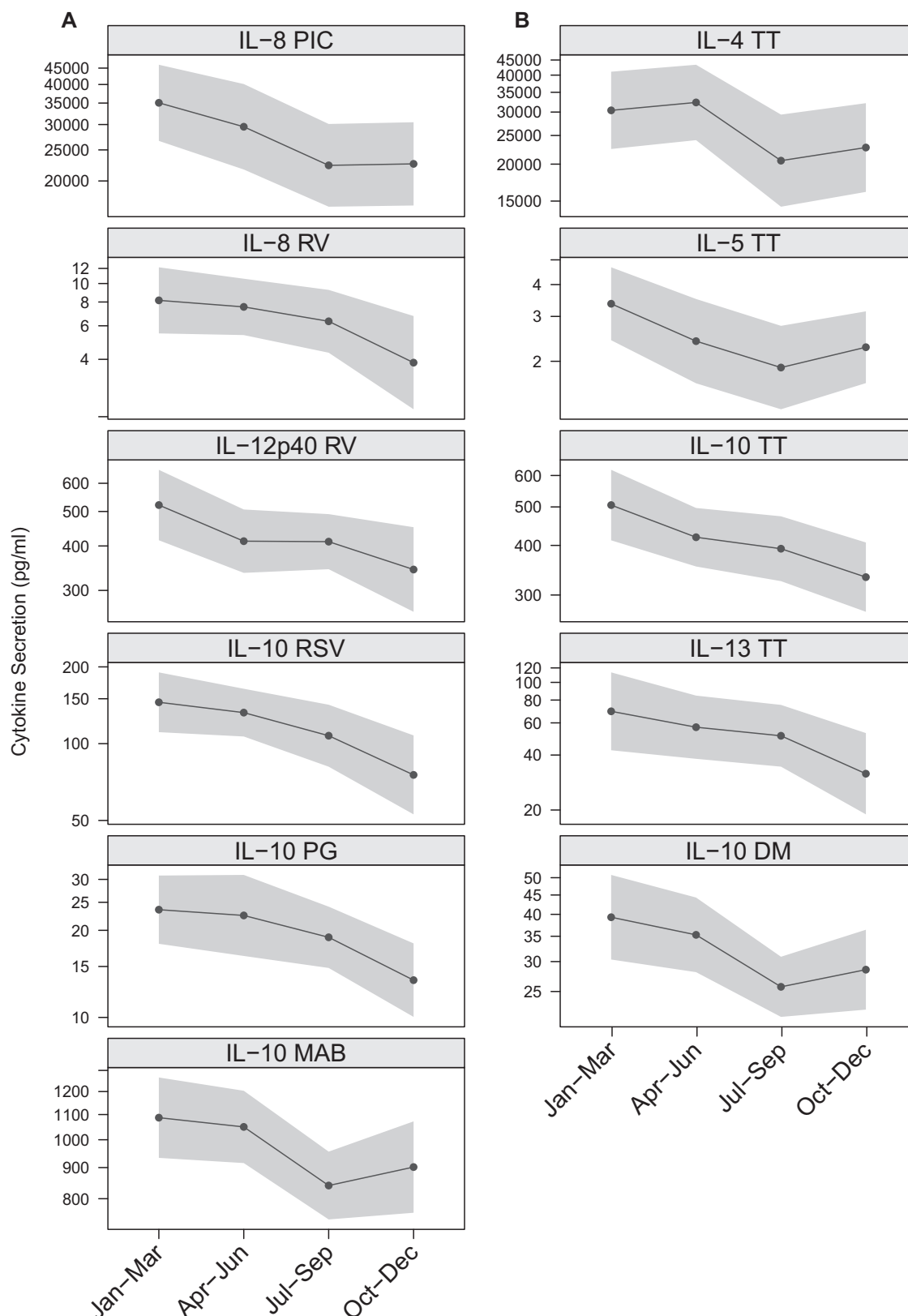
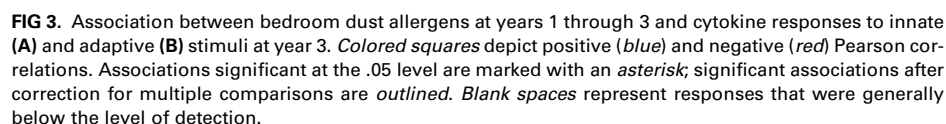


FIG 2. Associations between season of birth and cytokine responses at year 3. The graphs depict significant associations ($P < .05$) between season of birth and cytokine responses to innate and viral stimuli (**A**) and adaptive and polyclonal stimuli (**B**), and shaded areas represent the 95% confidence interval for the mean values.



The URECA study was conducted to test for relationships between early life environmental exposures and immune development as indicated by comprehensive measurements of PBMC cytokine responses and to identify patterns of immune

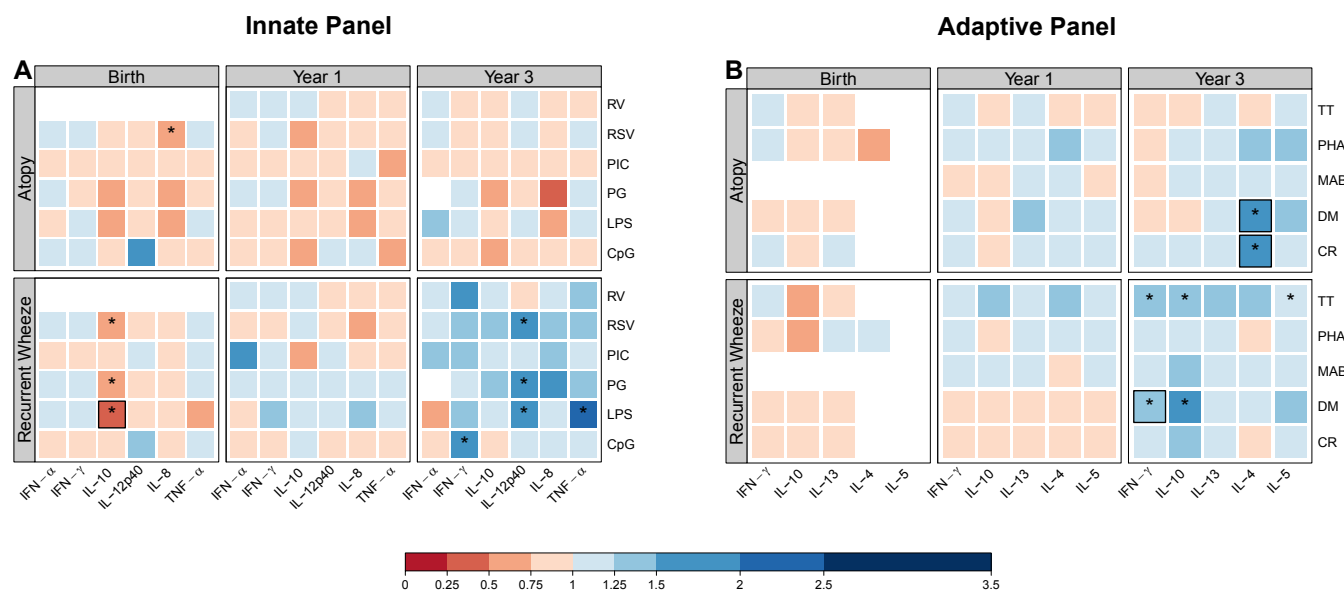


FIG 4. Association between cytokine responses at each year and outcomes at year 3. *Colored squares* depict positive (*blue*) and negative (*red*) odds ratios. Associations significant at the .05 level are marked with an *asterisk*; significant associations after correction for multiple comparisons are *outlined*. *Blank spaces* represent responses that were generally below the level of detection or were not measured.

TABLE II. Multivariate effects of cytokines on recurrent wheeze and atopy*

Stimulus and cytokine	CB birth		Year 1		Year 3		All years	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Recurrent wheeze								
LPS IL-10	0.50 (0.27–0.92)	.026					0.51 (0.27–0.96)	.038
LPS TNF-α					2.00 (0.93–4.31)	.076	2.00 (0.90–4.46)	.089
Atopy								
CpG IL-12p40	1.67 (1.05–2.67)	.030					1.88 (1.15–3.07)	.011
RSV IL-8	0.51 (0.30–0.87)	.013					0.51 (0.30–0.90)	.019
LPS IL-8			0.56 (0.30–1.05)	.071			0.54 (0.27–1.06)	.072
CR IL-4					1.57 (1.09–2.27)	.016	1.54 (1.06–2.25)	.024
DM IL-4					1.52 (0.99–2.32)	.055	1.55 (1.00–2.41)	.052

OR, Odds ratio.

*Cytokines that were selected >60 times in the LASSO regressions of the 100 training samples were entered as predictors in logistic regression models for the 50 test samples. Odds ratios and their associated confidence intervals and *P* values represent multiple-imputed estimates in the test samples. Cytokines were analyzed for each year separately and for all years combined. Models were adjusted for ethnicity and sex.

development associated with recurrent wheezing and allergic sensitization. We found that exposures in house dust to selected animal proteins (mouse, cockroach, and dust mite) and endotoxin were associated with enhanced cytokine responses at age 3 years, including some antiviral responses. In addition, personal characteristics such as ethnicity and season of birth were related to patterns of cytokine responses. Finally, distinct patterns of cytokine responses were associated with recurrent wheezing (IL-10 vs TNF responses to LPS) and allergic sensitization (selected IL-8 and IL-12p40 responses and increased IL-4 responses to allergens). Collectively, these findings provide insights into early life household exposures that could influence immune development in urban youth, and about the immunologic mechanisms that are associated with recurrent wheezing and allergic sensitization, 2 important risk factors for childhood asthma.

Associations between animal proteins and postnatal immune development have been noted in previous studies in distinct populations and environments. For example, exposure to dog proteins were associated with increased mitogen-induced IL-5, IL-10, and IL-13 at 1 and/or 3 years of age in the Childhood Origins of Asthma (COAST) birth cohort study¹³ and lower mitogen- and LPS-induced TNF-α responses at birth and age 1 year in Finnish infants.¹⁴ In urban environments, cockroaches are major allergens; however, we previously reported that URECA participants who had early life exposure to cockroaches (and to a lesser extent, mice and cats) were less likely to develop recurrent wheeze.¹¹ These findings suggested that exposure to cockroach might also have beneficial effects on immune development. In fact, exposure to cockroaches or endotoxin in URECA was associated with some of the same immunologic findings (increased PHA-induced cytokines) that were related to dog

exposure in the COAST study. Notably, cockroach and mouse exposure were also positively related to exposure to some interferon responses. The common clinical theme is that exposure to dog, endotoxin, and cockroach in the home have all been linked to reduced wheeze, suggesting that immunostimulatory properties of these exposures could be linked to reduced risk of recurrent wheeze.

Indoor dogs¹⁵ can increase the diversity of the home microbiome, and cockroaches, mice, and pets may have similar effects in urban homes. In a subset of URECA participants, we previously reported that children exposed to a diverse microbiome in their homes were less likely to develop atopy by age 3 years.¹¹ These findings raise the possibility that microbes associated with cockroaches, mice, and pets could mediate some of the observed associations with immune development. There was also evidence that allergens had effects that were independent of exposure to microbes, as early exposure to allergens, but not microbes, was inversely associated with recurrent wheeze.¹¹ Notably, neither cockroach nor dust mite content in settled dust were associated with T_H2-skewed responses to those allergens in the current analysis. These results suggest that allergenic proteins in the home environment stimulate immune development, and that other factors besides protein exposure (eg, heredity, microbial coexposures) contribute to antigen-specific T_H2 skewing in children who develop atopy.

Ethnicity influenced cytokine responses in URECA, and other studies have demonstrated that cytokine responses can be influenced by heredity.^{16,17} Despite these relationships, the network analysis in our study demonstrated no significant relationships between responses at birth and subsequent years. This finding is in agreement with observations that postnatal immune development is largely driven by nonheritable influences that are likely related to environmental exposures, such as herpesvirus infections and microbial exposures.¹⁸

Our analyses also identified patterns of cytokine development associated with recurrent wheeze and atopy; these relationships were outcome-specific. Recurrent wheeze was associated with low IL-10 responses to LPS at birth and enhanced TNF- α responses to LPS at age 3 years. Both viruses and bacteria in the airway contribute to wheezing illnesses,^{19,20} and our findings suggest that the quality of cytokine responses to gram-negative bacteria may be an important determinant of illness severity.

In contrast to associations with recurrent wheeze, atopy was inversely related to LPS- and RSV-stimulated IL-8 responses in early life and positively associated with development of enhanced IL-4 responses to allergens. IL-8 (chemokine [C-X-C motif] ligand 8) is a potent neutrophil chemoattractant, suggesting a potential link between the neutrophilic response to viral or bacterial infection and subsequent development of aeroallergen sensitization. The plausibility of this link is supported by evidence that circulating neutrophils have distinct features in allergic individuals,²¹ and neutrophils can promote the induction of Fc ϵ RI expression and allergy in animal models.²² The association of atopy with progressive T_H2 skewing to allergens was expected. An unexpected finding in the multivariate analysis is the positive association between CpG-induced IL-12p40 responses and allergic sensitization. IL-12p40 is a common subunit for IL-12p70, which promotes T_H1 differentiation, and IL-23, which maintains and expands T_H17 cells. Polymorphisms in the *IL12B* gene that encodes IL-12p40 have been linked to atopy

and lung function in children.^{23,24} Additional studies are needed to determine whether IL-12p40 responses in early life influence atopy and to determine potential mechanisms for this relationship.

Several previous studies have examined relationships between blood cell cytokine responses and the development of wheezing and atopy. In some studies, low interferon responses to mitogens or viruses in early life were associated with an increased risk for wheeze.^{25,26} In URECA, there were no significant associations between interferon responses at birth or age 1 and the development of wheezing or atopy. Of note, we previously reported in a subset of URECA infants that IFN- γ responses of monocytes (isolated from PBMC) were inversely related to several respiratory outcomes including prevalence of upper respiratory illnesses, ear and sinus infections, and respiratory hospitalizations.²⁷ These findings suggest that monocyte-lineage cell responses may be particularly important to regulation of wheezing illnesses, and this concept is consistent with findings from array-based measurements of PBMC during acute wheezing illnesses in asthma,²⁸ as well as findings in animal models of viral respiratory illness.²⁹

Several features of the study population and procedures should be considered in interpreting these data. The cytokine response assays were comprehensive and were conducted using freshly isolated cells in laboratories with careful quality control procedures and periodic assessments of technician performance and reproducibility.⁹ There were a large number of comparisons, which increases the risk of type 2 error; this risk was mitigated by the large sample size, statistical methods, and the testing of prespecified hypotheses. For practical reasons, environmental assessments were limited to 1 sample of settled dust per year. In addition, the URECA children are predominantly minorities living in disadvantaged neighborhoods, and all have a family history of allergies or asthma. Whereas this population is important to study due to increased incidence of allergy and wheezing illnesses, there may be different relationships in other populations. Furthermore, relationships between allergen exposure and the development of asthma could depend on context, including environmental microbes, presence of allergy, or community-level exposures. Finally, both atopy and recurrent wheeze in early life are likely to represent a mixture of different phenotypes that evolve with time, and there may be novel immunologic relationships with phenotypes or endotypes that become evident as we continue to follow these children.

In conclusion, findings of the URECA study provide evidence that immune development in urban infants is influenced by ethnicity, season of birth, and exposure to animal allergens and endotoxin. Notably, early life environmental exposures associated with reduced wheeze in URECA (eg, mouse, cockroach) and other studies (eg, endotoxin, dog) were associated with increased polyclonal response in URECA. These findings add to data from farming and suburban environments, indicating that exposure to animal proteins (including allergens) and microbes stimulate immune development in early life and suggest that these effects reduce the risk of recurrent wheezing.

Clinical implications: Patterns of immune development were significantly associated with exposure to allergens in early life and to the risk of developing allergic sensitization and recurrent wheezing.

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METHODS

Statistical analysis

LASSO automatically balances multivariate model performance against the number of predictors to identify the best set of cytokine responses associated with clinical outcomes. Cytokines that were selected in >60 of the 100 training datasets were entered as predictors to be validated in final logistic regression models. If no cytokine was selected >60 times, then the 1 cytokine selected most often among the 100 datasets was chosen as the predictor. Analyses were performed in SAS version 9.3 and in R version 3.1.1 (SAS Institute, Cary, NC). In particular, the imputation was created using the R

package MICE^{E1} and analyzed with SAS MIANALYZE^{E2} and the LASSO using glmnet.^{E3}

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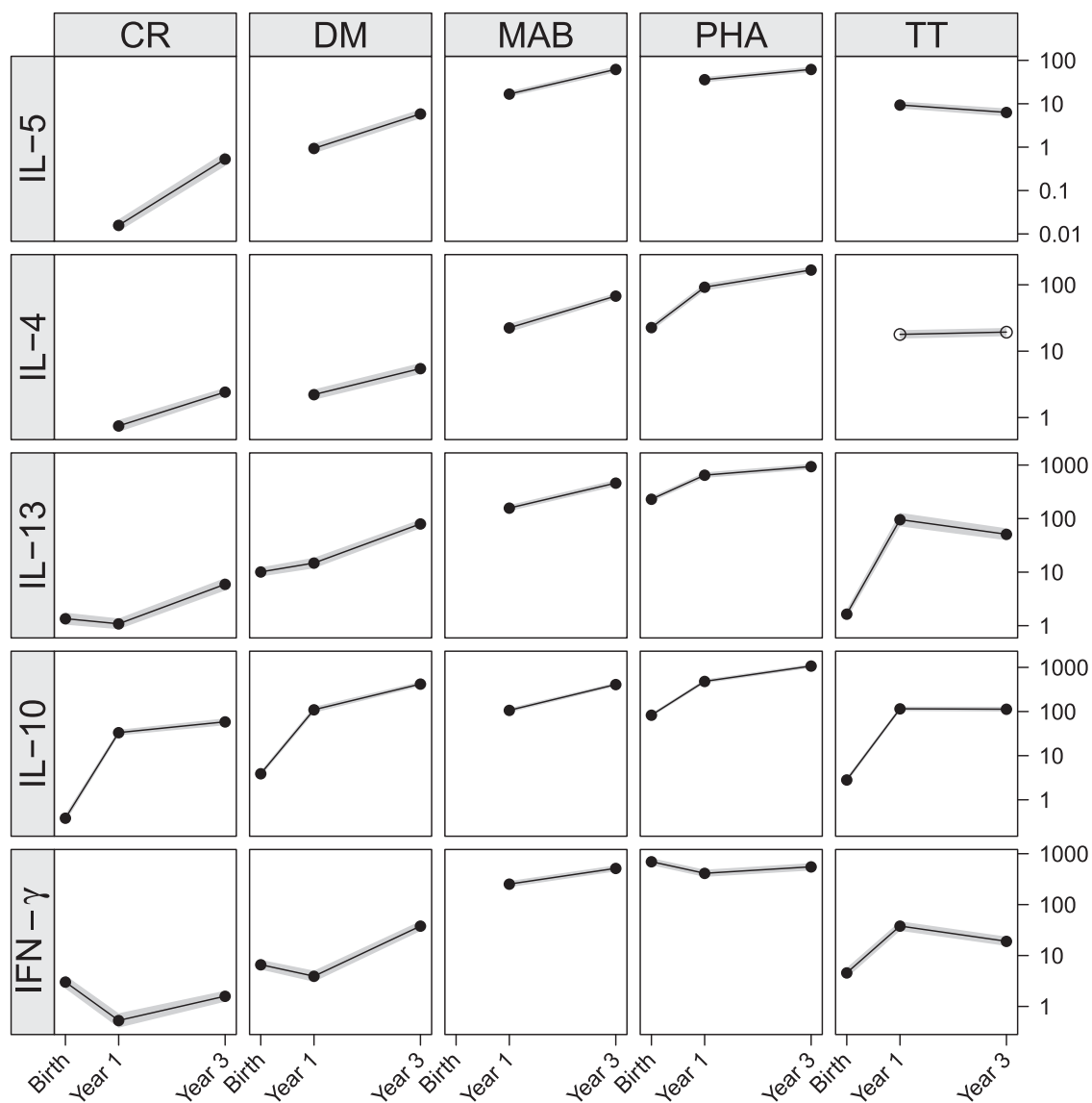


FIG E1. Developmental changes in cytokine responses to adaptive and polyclonal stimuli; average values for each time point. *Filled symbols* represent significant ($P < .05$) time-related changes in cytokine responses, and *shaded areas* represent the 95% confidence interval for the mean values.

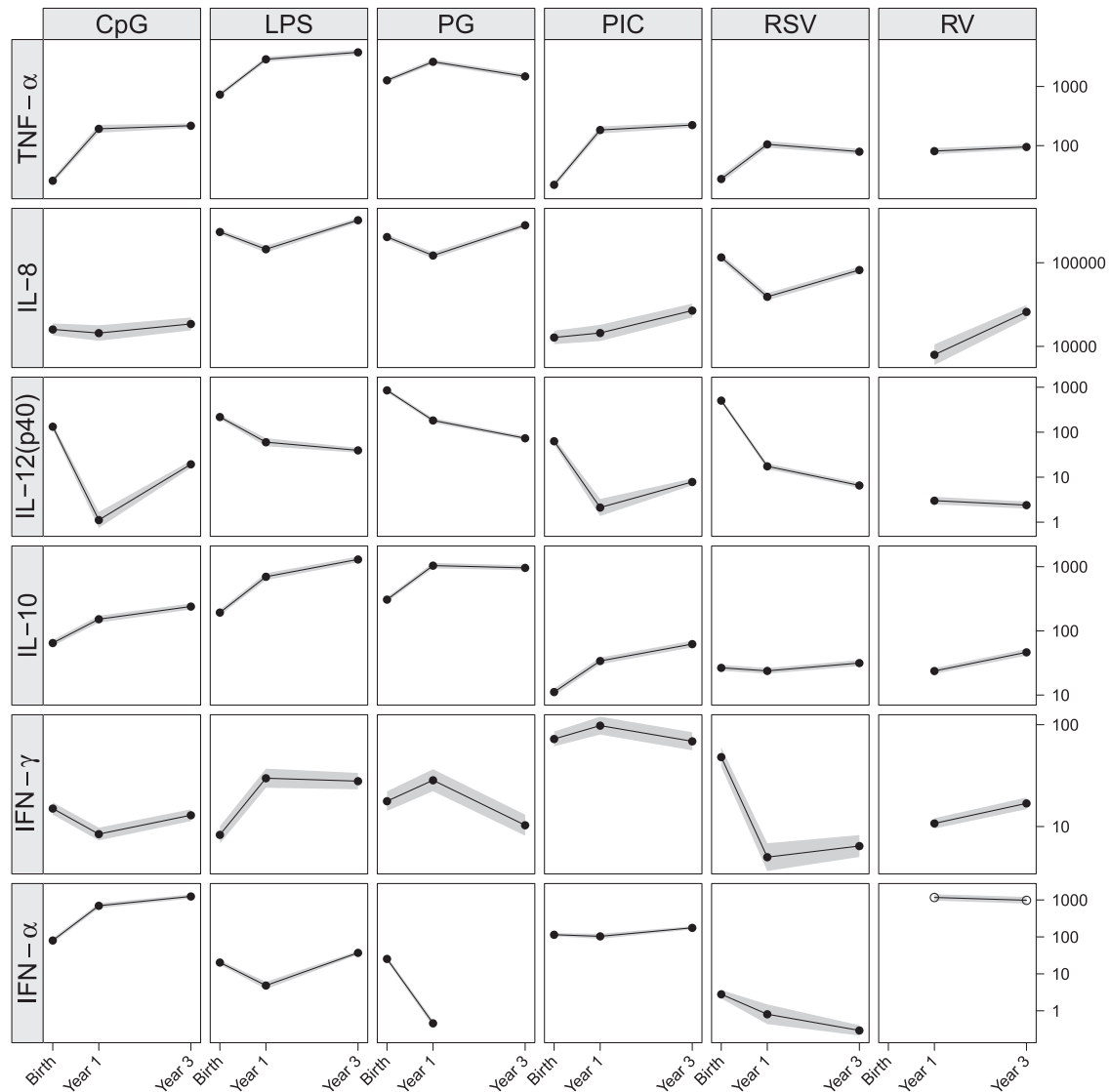


FIG E2. Developmental changes in cytokine responses to innate and viral stimuli; average values for each time point. Filled symbols represent significant ($P < .05$) time-related changes in cytokine responses, and shaded areas represent the 95% confidence interval for the mean values.

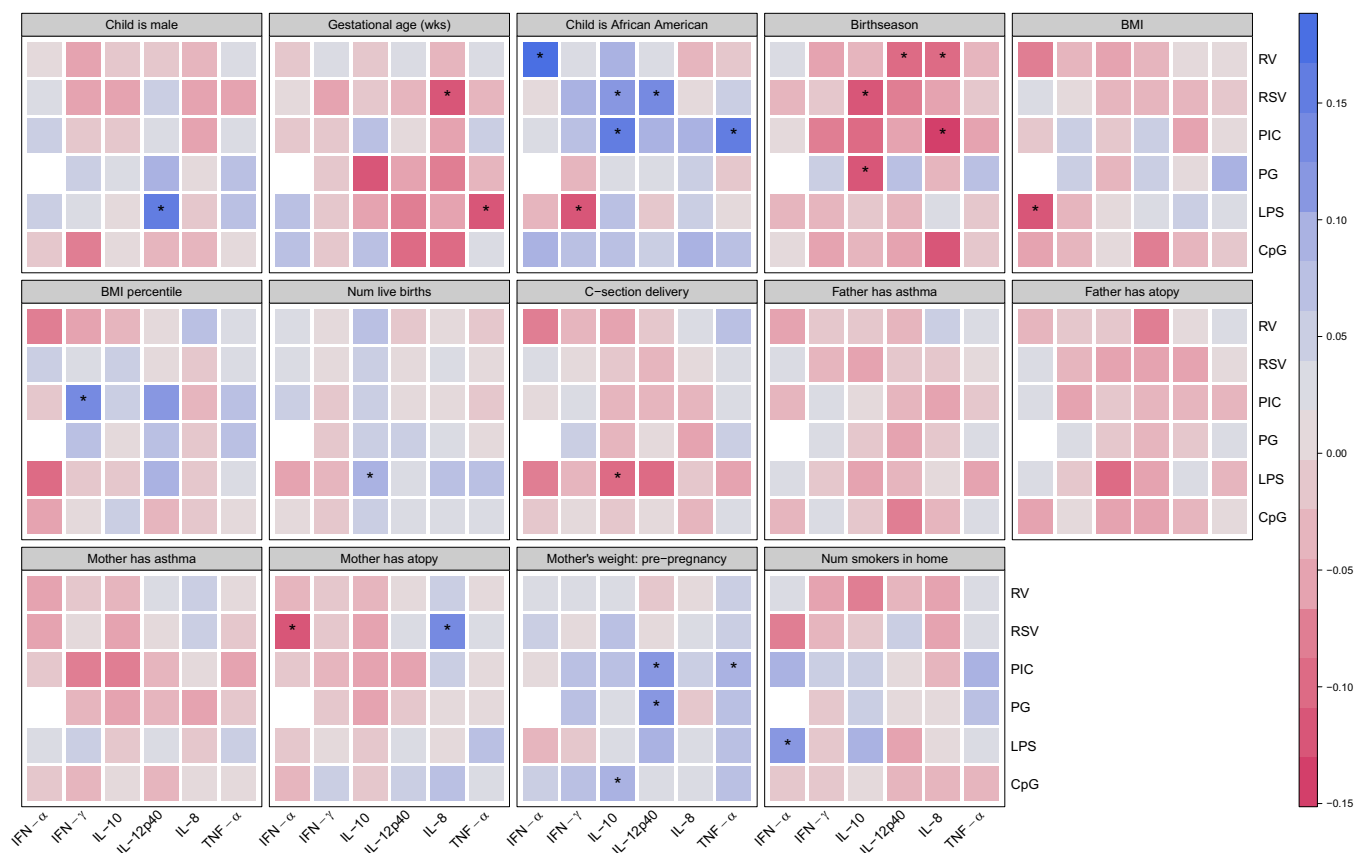


FIG E3. Univariate correlations between demographic data and cytokine responses (age 3 years) to innate stimuli. Each *square* depicts a Pearson correlation where *blue* shades represent positive correlations and *red* shades represent negative correlations. Associations significant at the .05 level are marked with an *asterisk*. *BMI*, Body mass index.



TABLE E1. Stimulants used and cytokines measured in mononuclear cell cytokine responses

Adaptive and polyclonal responses		Innate and antiviral responses	
Stimulants	Concentrations	Stimulants	Concentrations
LPS	0.1 µg/mL	PHA	15 µg/mL
PIC	25 µg/mL	CR	10 µg/mL
PG	1.25 µg/mL	DM	10 µg/mL
CpG	1 µg/mL	TT	5 µg/mL
RSV	500 sfu/mL	CD3 + CD28 mAb*	0.01 µg/mL
RV*	2.5×10^6 pfu/mL	Medium alone	
Medium alone			
Cytokines measured: IFN-α, IFN-γ, IL-8, IL-10, IL-12p40, TNF-α		Cytokines measured: IFN-γ, IL-4, IL-5, IL-10, IL-13	

pfu, Plaque-forming unit; *PIC*, polyinosinic-polycytidylic acid; *RV*, rhinovirus; *sfu*, syncytia-forming unit.

*Stimulation not conducted on cells from the umbilical cord samples.

TABLE E2. Demographic comparison of participants included versus excluded in analysis population

	Analysis population 83% (n = 467)	No age 3 follow-up data 17% (n = 93)	P value
Study site			.22
Baltimore	140 (30.0)	18 (19.4)	
Boston	108 (23.1)	24 (25.8)	
New York	93 (19.9)	22 (23.7)	
St. Louis	126 (27.0)	29 (31.1)	
Mother smoked during pregnancy	83 (17.8)	14 (16.1)	.82
No. of smokers in the home			.55
0	235 (50.5)	49 (56.3)	
1-2	203 (43.7)	35 (40.2)	
3+	27 (5.8)	3 (3.5)	
Mother has high school education	273 (58.6)	48 (55.2)	.64
Mother is married	64 (13.7)	8 (9.2)	.33
Mother has ever had asthma	234 (50.3)	36 (41.4)	.16
Age of mother at time of birth (y), mean $\pm$ SD	24.5 $\pm$ 5.9	23.8 $\pm$ 5.9	.30
Household income <\$15,000	321 (68.7)	48 (67.6)	.96
Child's ethnicity			.28
African American	333 (71.3)	56 (62.9)	
Hispanic	92 (19.7)	23 (25.8)	
Other/mixed	42 (9.0)	10 (11.3)	
Child's sex: male	240 (51.4)	47 (50.5)	.97
Caesarian delivery	154 (33.0)	21 (22.6)	.06
Gestational age of child at delivery (wks), mean $\pm$ SD	38.8 $\pm$ 1.5	38.4 $\pm$ 1.8	.04
Child's birth weight (g), mean $\pm$ SD	3258 $\pm$ 504	3131 $\pm$ 553	.04
Season child was born in			.72
January-March	113 (24.2)	24 (25.7)	
April-June	117 (25.1)	25 (26.9)	
July-September	138 (29.5)	22 (23.7)	
October-December	99 (21.2)	22 (23.7)	
Ever breastfed child	255 (56.0)	34 (68.0)	.14
Breastfed child in first 3 months	100 (23.9)	10 (22.2)	.95

Values are n (%) unless otherwise indicated.