Original Research Article

Microbial Exposures in Infancy Predict Levels of the Immunoregulatory Cytokine Interleukin-4 in Filipino Young Adults

PAULA SKYE TALLMAN,[1]* CHRISTOPHER KUZAWA,[1,2] LINDA ADAIR,[3] JUDITH B. BORJA,[4] AND THOMAS W. MCDADE[1,2]
[1]*Department of Anthropology, Northwestern University, Chicago, Illinois 60201*
[2]*Cells to Society: The Center on Social Disparities and Health at the Institute for Policy Research, Northwestern University, Chicago, Illinois 60201*
[3]*Department of Nutrition, UNC Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599*
[4]*USC Office of Population Studies Foundation, University of San Carlos, Talamban, Cebu City 6000, The Philippines*

Objectives: Infancy represents a window of development during which long-term immunological functioning can be influenced. In this study, we evaluate proxies of microbial exposures in infancy as predictors of interleukin-4 (IL-4) in young adulthood. IL-4 is an immunoregulatory cytokine that plays a role in the pathogenesis of atopic and allergic diseases.

Methods: Data were obtained from 1,403 participants in the Cebu Longitudinal Health and Nutrition Survey, an ongoing population-based study in the Philippines. Relationships between microbial and nutritional environments in infancy and plasma IL-4 concentrations in adulthood were evaluated using tobit regression models.

Results: Having older siblings and more episodes of respiratory illness in infancy significantly predicted lower concentrations of plasma IL-4 in adulthood. Unexpectedly, more episodes of diarrheal illness in infancy were associated with higher IL-4 in adulthood. Interactions between a composite household pathogen exposure score and the duration of exclusive breastfeeding approached significance. This interaction showed that the negative association between household pathogen exposure in infancy and adult IL-4 was only significant for individuals who had been exclusively breastfed for a short duration of time. Finally, currently living in an urban household was unexpectedly, negatively associated with adult IL-4. Associations were independent of early nutrition, socioeconomic status (SES), and urbanicity, as well as current measures of infection, body fat, SES, and smoking.

Conclusions: This study builds on a growing body of literature demonstrating that early ecological conditions have long-term effects on human biology by providing evidence that multiple proxies of microbial exposures in infancy are associated with adult IL-4. Am. J. Hum. Biol. 00:000–000, 2012.

Substantial evidence indicates that prenatal and postnatal environments have long-term effects on human health (Barker et al., 1989; Gluckman et al., 2007, 2008; Power and Hertzman, 1997; Prentice and Moore, 2005) and may contribute to organismal adaptation (Gluckman et al., 2007; Kuzawa and Pike, 2005; Kuzawa and Quinn, 2009). Although the majority of work on early physiological programming has been focused on the cardiovascular and endocrine systems (Barker et al., 1993; Breier and Gluckman, 1991; Gluckman and Hanson, 2006; Godfrey, 2006), an emerging body of evidence in human ecological immunology suggests that the effects of the early environment also extend to the immune system (Godfrey et al., 1994; McDade, 2003b, 2005; McDade and Worthman, 1999; Phillips et al., 1993). More specifically, immunologists and epidemiologists researching the "hygiene hypothesis" have found that early microbial exposures are linked with adult immune function (Strachan, 2000; von Mutius, 2007).

The hygiene hypothesis proposes that a suite of sociodemographic and environmental changes in affluent industrialized settings have reduced microbial exposures early in life, leading to perturbations in immune development and increases in the expression of atopy and allergy (Garn and Renz, 2007; Strachan, 1989). Atopy and allergy are immunological diseases that include asthma, allergic rhinitis, and eczema and are often characterized by elevated levels of immunoglobulin E (IgE) in response to common allergens (Johansson et al., 2001). Although several studies have measured levels of IgE in relation to early microbial exposures (Douwes et al., 2006; Matricardi et al., 1998; McDade et al., 2004; Riedler et al., 2001), little attention has been paid to the upstream regulators of IgE such as the cytokine interleukin-(IL)-4.

IL-4 is a prototypic immunoregulatory cytokine that is secreted by activated T lymphocytes, basophils, and mast cells (Brown and Hural, 1997; Hou et al., 1994; Paul, 1991). Although IL-4 plays a critical role in the regulation of the adaptive immune response, in the induction and regulation of IgE, and in the pathogenesis of atopic disease, the majority of studies of IL-4 have been conducted in animal models (Finkelman et al., 2004; Kudsk et al., 2000) and in clinical populations (Dlugovitzky et al., 1997; Fraser et al., 1999; Giron-Gonzalez et al., 2000; Hauer, 1997; Hou et al., 1994; Pène et al., 1988; Reiser et al., 1997; Stoeck et al., 2005; Tang et al., 1993; van Crevel

Contract grant sponsor: National Institutes of Health; Contract grant numbers: RO1 HL085144, 5 RO1 TW05596; Contract grant sponsor: Interdisciplinary Obesity Center; Contract grant number: RR20649; Contract grant sponsor: Center for Environmental Health and Susceptibility; Contract grant number: ES10126; Contract grant sponsor: National Science Foundation.

*Correspondence to: Paula Skye Tallman, Department of Anthropology, Northwestern University, Chicago, IL 60201, USA.
E-mail: paulaskye@u.northwestern.edu

Received 27 September 2011; Revision received 12 December 2011; Accepted 27 December 2011

DOI 10.1002/ajhb.22244

Published online in Wiley Online Library (wileyonlinelibrary. com).

et al., 2000; Wong et al., 2000). Consequently, little is known about normal circulating levels of IL-4 in nonclinical human populations. Even less is known about factors in the early environment that may be associated with the expression of IL-4 in adulthood.

The objective of this work is to evaluate the associations between proxy measures of microbial exposures in infancy and IL-4 concentrations in a representative community-based sample of young adults. We hypothesized that increased early microbial exposures—indicated by respiratory and diarrheal infections in infancy, later birth order, larger household size, and exposure to animal and human waste—will be associated with lower IL-4 in adulthood. We also considered prenatal and early postnatal nutritional factors that have been shown to influence immune development and function (Godfrey et al., 1994; McDade et al., 2001; Phillips et al., 1993), including birth weight, preterm delivery, and duration of exclusive breastfeeding. We explicitly tested for interactions between exclusive breastfeeding and microbial exposure due to the important buffering and immune-modulating role of breast milk early in infancy (Field, 2005; McDade and Worthman, 1998; Oddy, 2001) and research demonstrating that pathogenic exposures, length of time spent exclusively for breastfeeding, and frequency of diarrheal disease are linked (VanDerslice et al., 1994). Finally, we included infant (1983) and adult (2005) measures of socioeconomic status (SES) and urbanicity based on epidemiological evidence showing that there are SES gradients and rural–urban differences in the expression of allergic and atopic diseases (Godfrey, 1975; Merrett et al., 1976; Strachan, 2000).

Data and samples were obtained from participants in an ongoing birth cohort in and around Cebu City, the Philippines, who have been followed prospectively since the third trimester of pregnancy into early adulthood. Currently, the reported prevalence of allergy in the Philippines is low (Warner et al., 2006); however, rates of asthma in urban areas are on the rise (Zainudin et al., 2005). The combination of low, but rising levels of atopy/allergy, heterogeneity in environmental conditions and prospectively collected information on microbial exposures in infancy provides a unique opportunity to investigate how ecological factors may shape the development and function of an important aspect of human immunity.

METHODS

The Cebu Longitudinal Health and Nutrition Survey began in 1983 with enrollment of 3,327 pregnant women and continues to collect data from the offspring to the present. Initial data collection efforts included home visits that were conducted in the last trimester of pregnancy, immediately following birth, and every 2 months for the following 2 years. During this time, data on anthropometrics, patterns of breastfeeding, illness episodes, household demographics, and environmental quality were collected. Several follow-up surveys were conducted thereafter. In the 2005 survey, when participants were on average 20.94 years old, data collection included venipuncture blood samples (Adair et al., 2011) that were used here for cytokine analysis.

Data for both early microbial environments (1983) and adult IL-4 concentrations (2005) were available for 1,403 male and female participants. When compared with individuals lost to follow-up, the participants in this study had higher mean birth weight (3007 vs. 2974 g, $P < 0.05$), were born to mothers with slightly less formal education (7.27 vs. 7.80 years, $P < 0.001$), experienced more infections over their first year of life (4.59 vs. 3.97, $P < 0.001$), were born into households with less material assets (summary mean 2.42 vs. 2.59, $P < 0.05$), and were more likely to live in a rural area. Household income and household size for individuals included in this study did not significantly differ from participants who were lost to follow-up.

Independent variables

Following prior research on the hygiene hypothesis (Matricardi, 1997; Riedler et al., 2001; Shaheen et al., 1996; Strachan, 1989; von Mutius et al., 1994), four measures of microbial exposures were considered: household size, the number of infections experienced during the first year of infancy, the presence of older siblings, and household pathogen exposures. First, household size included persons present and persons absent from the household during the baseline survey in 1983. This measure was categorized as follows: very small (<2), small (3–4), medium (5–6), large (7–8), and very large (>9) based on the number of people reported to be living in the house.

Second, to obtain measures of early infection, mothers reported whether their child had experienced diarrhea or a respiratory illness episode in the week before the bimonthly interviews. To ascertain whether the child had had a respiratory illness, mothers were given an example of a respiratory illness episode, which included symptoms such as cough, fever, nasal congestion/discharge, ear discharge, and sore throat, and could respond that the child had an episode in the past week (1) or did not (0). A variable indicating the frequency of respiratory illness episodes over the first year of life was created by summing the total number of interviews when a respiratory illness was reported (possible range: 0–6). A similar variable was constructed for diarrheal episodes (possible range: 0–5).

Third, we defined the presence of older siblings using a dichotomous variable, with 1 indicating that the participant was firstborn (no older siblings), and 0 indicating the presence of one or more older siblings. A child without older siblings would likely have reduced chances of cross-infection and thus lower microbial exposures in infancy (Matricardi et al., 1998; Strachan, 1989).

Finally, a household pathogen exposure score was constructed, which included the following measures based on interviewer observations of the home at baseline in 1983: (1) the presence of domestic animals beneath the house (2 = always, 1 = sometimes, 0 = never); (2) the presence of excrement around the house (2 = heavy, 1 = some, 0 = none); (3) an unhygienic food storage area (2 = filthy, 1 = not so clean, 0 = very clean); and (4) type of toilet used (2 = field, 1 = latrine, 0 = flush/water sealed). These variables were summed to create a household pathogen exposure score that ranged from 0 (lowest exposure) to 8 (highest exposure).

Additional variables collected at baseline in 1983 were also considered, including SES [measured by maternal education (years), household assets (summary score), and household income (pesos)], and whether the home was in an urban area or not [7-component urbanicity scale derived from Dahly and Adair (2007)]. These variables were included based on findings that parental SES (early

TABLE 1. Sample characteristics of 1,403 young adult males and females

	Males	Females	Both
Variables assessed in infancy			
Birth weight (kg)	3.03 (0.42)	2.98 (0.42)	3.00 (0.42)
Weight gain, first year (kg)	5.19 (0.92)	4.68 (0.88)	4.95 (0.93)
Mothers' education (years)	7.40 (3.63)	7.14 (3.47)	7.28 (3.56)
Household income (pesos)	297.34 (604.00)	256.90 (318.57)	278.32 (491.01)
Household size	5.70 (2.78)	5.70 (2.82)	5.70 (2.80)
Household pathogen exposure	3.48 (1.80)	3.42 (1.74)	3.45 (1.77)
Episodes of respiratory infection, first year	4.40 (1.32)	4.25 (1.38)	4.32 (1.35)
Episodes of diarrhea, first year	1.18 (1.14)	1.03 (1.02)	1.11 (1.09)
Variables assessed in adulthood			
Age (years)	20.94 (0.33)	20.93 (0.35)	20.94 (0.34)
Education (years)	10.44 (3.81)	11.37 (3.19)	10.88 (3.57)
Body mass index	21.04 (3.04)	20.52 (3.26)	20.79 (3.15)
IL-4 (median, 25th and 75th percentile; pg/ml)	1.96 (0.001, 7.35)	1.69 (0.001, 6.80)	1.81 (0.001, 7.18)
IL-4 (mean, SD; pg/ml)	8.89 (60.69)	7.50 (39.27)	8.23 (51.71)

Values are expressed as mean (SD).

SES) was associated with the development of atopic disease independently of birth order, family size, and early respiratory infections (Forastiere et al., 1997; Strachan, 1995).

Variables reflecting the early nutritional environment included (1) time spent exclusively for breastfeeding (median duration = 57 days; short breast-feeders < 57 days, long breast-feeders > 57 days); (2) birth weight (kilograms); (3) weight gained during the first year (grams); and (4) gestational age at birth. Gestational age was indicated by whether the individual was born preterm or not (0 or 1), which was determined using the date of last menstrual period and the Ballard method in complicated pregnancies (Adair et al., 2011).

Variables measured in adulthood included body mass index (BMI; kg/m^2), sex (male or female), daily smoking (yes or no), SES [measured by highest level of education attained (years), household assets (summary score), and household income (pesos)], urbanicity, and whether participants had any symptoms of infection at the time of blood collection (cough, fever, nasal congestion/discharge, ear discharge, sore throat, or diarrhea).

IL-4 analysis

Blood samples were collected in the home using Ethylenediaminetetraacetic acid (EDTA)-coated vacutainer tubes after an overnight fast. Once collected, blood samples were preserved in coolers on ice packs for less than 2 h, transported to a central facility, centrifuged to separate plasma, and then frozen at −70°C. Samples were express shipped on dry ice to the Northwestern University and stored at −80°C until analysis. Plasma concentrations of IL-4 were determined using a high-sensitivity multiplex immunoassay protocol (HSCYTO-60SK; Millipore, Billerica, MA) on the Luminex platform (Luminex Corporation, Austin, TX). Interassay coefficients of variation for low and high control samples were 22.2% and 16.3%, respectively. The assay lower detection limit was 0.12 pg/ml. Samples below this limit were assigned a value of 0.001 pg/ml.

Data analysis

The analyses proceeded in three stages. First, descriptive analyses were used to examine mean and median levels of adult IL-4 for males and females. Second, bivariate associations were examined between log-transformed IL-4 and the primary independent variables of interest, which included measures of household size, infectious morbidity, household pathogen exposure, and having older siblings. Interactions between exclusive breastfeeding duration and each of these variables were then considered based on work showing that the protective effects of breastfeeding vary based on the pathogenic environment (VanDerslice et al., 1994).

Third, multivariate models were adjusted for (1) early measures of SES, (2) prenatal (birth weight, preterm delivery) and postnatal factors (weight gain over the first year), which likely correlate with microbial exposure, and (3) factors in adulthood (BMI, symptoms of infectious disease, sex, smoking status, SES and urbanicity) that could possibly confound associations between IL-4 and microbial exposures in infancy. Here, a confounder is defined as any variable that leads to a change of 10% or greater in the effect of the main predictors.

An α-value of <0.05 was considered the criterion for statistical significance, with $P < 0.10$ indicating trends in the data. All statistical analyses were conducted with Stata for Windows, version 10 (StataCorp, College Station, TX). We applied tobit regression models for censored data to account for non-normality in the distribution of the IL-4 values (seen in Figure 1). Left censoring of the distribution is due to the large number of observations with values below the lower detection limit of the cytokine assays. Applying ordinary least squares regression resulted in similar associations; however, tobit regression provides more reliable parameter estimates in this case (Greene, 2000).

RESULTS

Basic descriptive statistics for males and females are presented in Table 1. The median IL-4 concentration for the entire sample was 1.81 pg/ml (interquartile range = 0.001–7.18). The mean IL-4 concentration was 8.23 pg/ml (SD = 51.71). Differences between mean and median values are likely a result of the skewed distribution of the IL-4 values. Figure 1 presents a histogram of this distribution. There were no significant differences in concentrations of IL-4 between males and females. Of these participants, 39% had undetectable concentrations of IL-4 (<0.12 pg/ml).

Bivariate associations (Table 2) revealed a significant negative association between the number of episodes of respiratory illness over the first year of life and IL-4 in adulthood, as well as a significant positive association

between the number of episodes of diarrheal illness over the first year of life and IL-4 in adulthood. Additionally, there was a positive association between being a firstborn child and IL-4 in adulthood. Household size and the summary household pathogenicity variable were not significant predictors of adult concentrations of IL-4. However, there was evidence for a marginally significant interaction between the level of household pathogen exposure and the duration of exclusive breastfeeding, strengthening the association between early life pathogen exposure and adult IL-4 for short-term breast-feeders.

Our first multivariate model (Table 3, Model 1) included measures of SES and urbanicity in infancy to investigate potential confounding of the association between early microbial exposures and IL-4. Measures of SES and urbanicity in infancy were not significant predictors of IL-4, and coefficients for microbial exposures did not change by more than 10%. Next, we considered whether the associations between microbial exposures and IL-4 were independent of preterm birth, birth weight, and weight gain over the first year of life (Table 3, Model 2). Associations between microbial exposure variables and IL-4 remained similar, with the exception of firstborn status, which strengthened as a predictor after further adjustment. Being born preterm was a marginally significant predictor of lower IL-4 in adulthood.

Finally, the third multivariate model included current BMI, illness, SES and urbanicity (Table 3, Model 3). Again, the addition of these variables did not result in

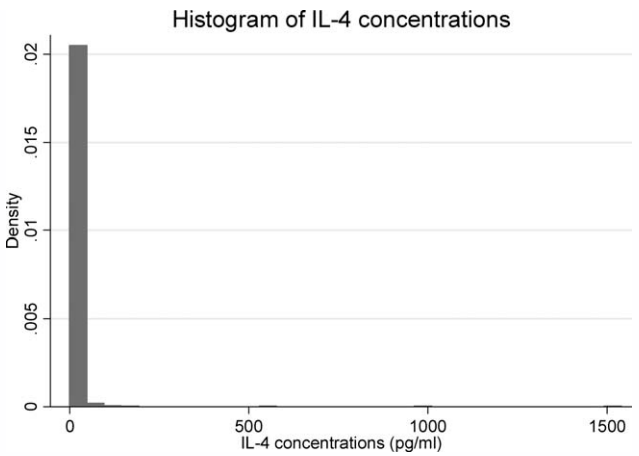

Fig. 1. Histogram of IL-4 concentrations demonstrating skewed distribution.

TABLE 2. Tobit regression analysis of bivariate associations between microbial exposures in infancy and log IL-4 in young adulthood

Bivariate associations	β (SE)	*P*
Microbial exposures in infancy		
Episodes of respiratory infection, first year	−0.45 (0.17)	0.01**
Episodes of diarrhea, first year	0.38 (0.21)	0.07†
Firstborn status	1.14 (0.54)	0.04*
Household pathogen exposure	−0.09 (0.13)	0.51
Household size	0.28 (0.19)	0.15
Duration of exclusive breastfeeding	0.07 (0.46)	0.87
Interactions		
Duration exclusive breastfeeding × respiratory illness	−0.04 (0.34)	0.91
Duration exclusive breastfeeding × diarrhea	0.65 (0.42)	0.12
Duration exclusive breastfeeding × firstborn	0.32 (1.08)	0.77
Duration exclusive breastfeeding × pathogen	0.43 (0.26)	0.10†
Duration exclusive breastfeeding × household size	−0.25 (0.39)	0.51

*$P < 0.05$.
**$P < 0.001$.
†$P < 0.10$.

TABLE 3. Tobit regression analysis of multivariate associations between microbial exposures in infancy and log IL-4 in young adulthood

	Model 1		Model 2		Model 3	
Multivariate associations	β (SE)	*P*	β (SE)	*P*	β (SE)	*P*
Infancy						
Household size	0.26 (0.21)	0.21	0.26 (0.21)	0.20	0.25 (0.21)	0.22
Household pathogen exposure	−0.24 (0.21)	0.21	−0.23 (0.21)	0.26	−0.26 (0.21)	0.21
Episodes of respiratory infection, first year	−0.54 (0.17)	0.00**	−0.53 (0.18)	0.00**	−0.50 (0.18)	0.01**
Episodes of diarrhea, first year	0.53 (0.22)	0.02*	0.53 (0.22)	0.02*	0.53 (0.22)	0.02*
Firstborn	1.09 (0.55)	0.05*	1.29 (0.57)	0.03*	1.32 (0.57)	0.02*
Duration of exclusive breastfeeding	1.21 (1.00)	0.23	−1.21 (1.00)	0.23	−1.18 (1.00)	0.24
Interaction between duration of breastfeeding × pathogen	0.38 (0.26)	0.15	0.37 (0.26)	0.15	0.37 (0.26)	0.15
Maternal education	−0.03 (0.08)	0.74	−0.03 (0.08)	0.73	−0.08 (0.08)	0.36
Household assets	0.00 (0.16)	0.95	0.00(0.16)	0.99	−0.02 (0.14)	0.90
Household income	0.00 (0.00)	0.45	0.00 (0.00)	0.47	0.00 (0.00)	0.59
Urban household	0.01 (0.02)	0.78	0.01 (0.02)	0.80	−0.05 (0.02)	0.17
Birth weight			0.42 (0.56)	0.45	0.27 (0.57)	0.64
Weight gain, first year			0.00 (0.00)	0.86	0.00 (0.00)	0.46
Preterm			−1.24(0.71)	0.08†	−1.15 (0.71)	0.10†
Adulthood						
Education, 2005					0.03 (0.07)	0.74
Assets, 2005					0.10 (0.14)	0.47
Household income, 2005					0.00 (0.00)	0.09†
Urban household, 2005					−0.05 (0.02)	0.04*
Smoker					0.17 (0.62)	0.79
Sick now					−0.03 (0.51)	0.96
Body mass index, 2005					0.09 (0.08)	0.24
Sex					−0.29 (0.55)	0.60

*$P < 0.05$.
**$P < 0.001$.
†$P < 0.10$.

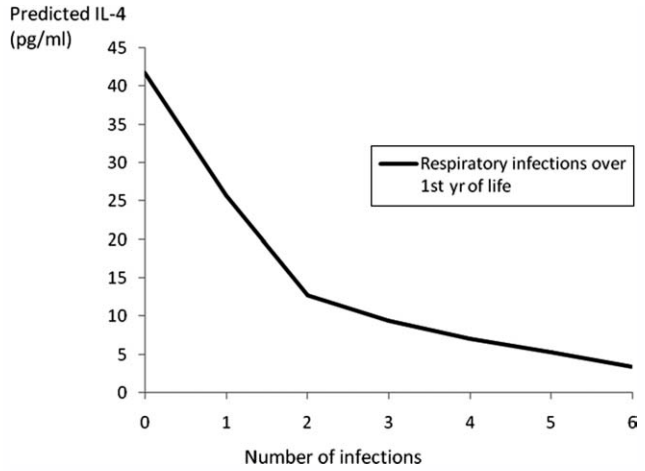

Fig. 2. Association between the number of respiratory infections in infancy and IL-4 in young adulthood. Coefficients from Table 3 (Model 3) were used to generate predicted log IL-4 values, which were then untransformed.

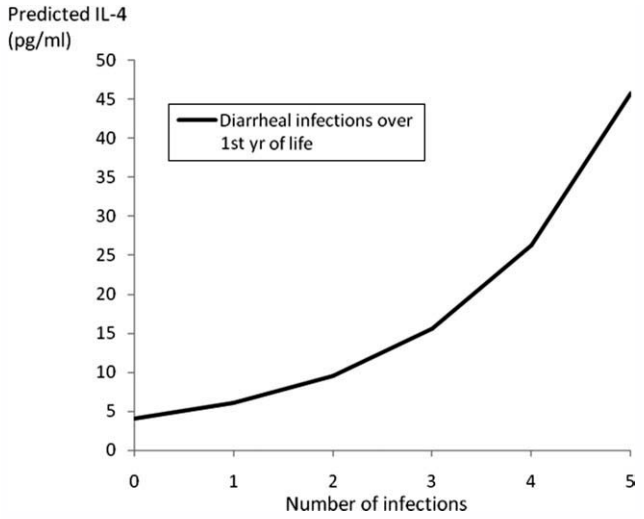

Fig. 3. Association between the number of diarrheal episodes in infancy and IL-4 in young adulthood. Coefficients from Table 3 (Model 3) were used to generate predicted log IL-4 values, which were then untransformed.

more than a 10% change in the effects of the main predictors on IL-4. Thus, we concluded that these variables were not confounding. We chose not to remove these variables from the final model because their inclusion revealed a significant negative association between adult IL-4 and current urbanicity, as well as associations between IL-4 and current household income that approached significance. Although these associations were very weak and did not significantly modify associations between firstborn status or infectious morbidity and adult IL-4, the final model retains these variables.

To summarize, we found four significant associations in our final model (Model 3). First, there is a significant negative association between the number of episodes of respiratory illness in the first year of life and adult IL-4. Second, there is an unexpected, and significant, negative association between the number of diarrheal episodes in the first year of life and adult IL-4. Third, there is a significant positive association between being the firstborn child and adult IL-4 such that having older siblings predicts a low IL-4 concentration of 4.90 pg/ml, whereas being a firstborn child predicts a slightly elevated IL-4 concentration of 14.33 pg/ml. Finally, there is an unexpected, and significant, negative association between currently living in an urban area and IL-4. With a β-coefficient of 0.00, the relationship between current household income and IL-4, which approaches significance, is realistically meaningless. On the other hand, the strength of the negative association between being born preterm and adult IL-4, which approaches significance, warrants further discussion.

Figure 2 presents the predicted values of IL-4 in relation to the number of episodes of respiratory illness during the first year of life. An individual with "0" reported respiratory illness episodes has a predicted concentration of 41.82 pg/ml, whereas a child with "6" respiratory illness episodes has a predicted concentration of 3.35 pg/ml. Figure 3 presents the predicted values of IL-4 in relation to the number of diarrheal episodes, demonstrating the opposite relationship, "0" episodes predicted a low concentration of 4.02 pg/ml of IL-4 and "5" episodes predicted a high concentration of 45.79 pg/ml.

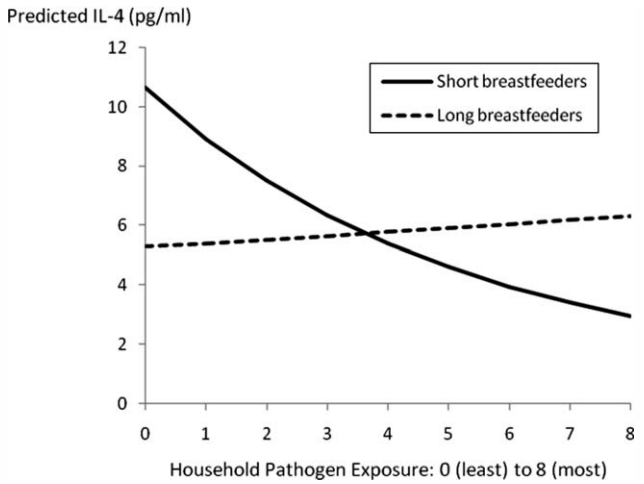

Fig. 4. Interaction between household pathogen exposure score and duration of exclusive breastfeeding in predicting IL-4 in young adulthood. Coefficients from Table 3 (Model 3) were used to generate predicted log IL-4 values, which were then untransformed. These values were not statistically significant.

Although the interaction between exclusive duration of breastfeeding and household pathogen exposure was of marginal statistical significance, we explored these associations further in fully adjusted models that were run separately for short- and long-term exclusive breast-feeders. We found large differences in the direction and strength of the associations for long- and short-term breast-feeders. Figure 4 plots the predicted values for both groups, showing that lower pathogen exposures only predicted higher IL-4 for short-term exclusive breast-feeders.

DISCUSSION

This study is the first to investigate circulating levels of plasma IL-4 in a large nonclinical sample of young adults and to examine early life predictors of adult IL-4. We find

that multiple proxies of microbial exposures in infancy predict lower IL-4 in adulthood. These findings are consistent with predictions generated by prior work on the hygiene hypothesis and extend our understanding of the physiological pathways that may be linking human ecologies with later immune function.

Immune disorders such as asthma, eczema, and rhinitis are characterized by increases in Th2 immune cell responses and in the production of IgE (Johansson et al., 2001; Romagnani, 2004). The hygiene hypothesis provides extensive evidence showing that early microbial exposures are associated with the expression of these immune disorders and with concentrations of IgE in adulthood. Although we know that IL-4 is associated with increased IgE and the expression of atopy in humans (Del Prete et al., 1988; Finkelman et al., 1988; Magnan et al., 2000; Pène et al., 1988), all studies of IL-4 have relied on stimulated or *in vitro* measurements, leaving our understanding of the regulation of IL-4 *in vivo* incomplete.

The negative association between respiratory infections in infancy and IL-4 in young adulthood in this study is consistent with prior research reporting that early infections are associated with lower concentrations of IgE later in life (Martinez et al., 1995; McDade et al., 2004). In addition, the separate and significant positive association found in this study between being the firstborn child and adult IL-4 is consistent with previous work reporting that individuals without older siblings had a higher prevalence of atopy, as defined by high levels of specific IgE against inhalants (Matricardi et al., 1998). Our findings support the basic premise of the hygiene hypothesis, which states that variation in immunological functioning is a reflection of differential exposure to pathogens early in life (Strachan, 2000), and make a strong case for the hypothesis that external ecologies that structure microbial exposures in infancy are related to the production of IL-4 and IgE later in life.

The positive association between diarrheal infections and IL-4 runs counter to our initial hypotheses. One potential explanation is that factors influencing the initial establishment of beneficial microflora in the infant's intestine are more important than infections per se in protecting against the development of atopy and allergy (Rautava et al., 2004). It is therefore possible that infants with more diarrheal episodes lack the beneficial gut microbiota, which would prevent diarrhea and contribute to healthy immunological maturation, possibly explaining why infants with more diarrheal episodes have higher IL-4 in adulthood in our sample.

Consistent with this possibility, short duration of exclusive breastfeeding is associated with higher rates of diarrhea in this sample (VanDerslice et al., 1994) and higher mean IL-4 levels in adulthood. Breastfeeding has been consistently shown to influence fecal flora composition (Stark and Lee, 1982; Yoshioka et al., 1983) and to protect infants against diarrheal disease in all environments (VanDerslice et al., 1994). Although speculative, our finding that the association between household pathogen exposure and adult IL-4 only holds for short-term exclusive breast-feeders suggest that long-term exclusive breastfeeding may buffer infants from external microbial environments. This could be occurring because breastfeeding aids in the establishment of beneficial gut microbiota (Rinne et al., 2005) and decreases contact with waterborne and foodborne pathogens (Popkin et al., 1990).

It is not clear why preterm delivery was independently associated with lower IL-4 in our sample. One possibility is that the functional immaturity of the neonatal immune system may predispose premature infants to increased episodes of infection early in life (Sadeghi et al., 2007). Another possibility is based on evidence showing that preterm birth is consistently linked with infections of the urogenital tract (Reid and Bocking, 2003) and with periodontitis (Dörtbudak et al., 2005), suggesting that babies born preterm could have higher exposure to microbes *in utero* and during delivery. This possibility draws attention to the need for future research to consider the potential role of prenatal microbial exposures in immune system programming (Prescott, 2003).

Prenatal programming of the immune system could also be potentially contributing to the association we found between birth order and adult IL-4. Although we assume that firstborn status represents a reduced level of microbial exposure in infancy, because of lack of infection from older siblings, there is some evidence that the sibling effect has its origins *in utero* (Karmaus et al., 2001). For example, the *in utero* programming disruption hypothesis links maternal exposure to allergens and the immunological changes that occur during pregnancy with the prenatal origins of allergies, whereas other *in utero* programming concepts focus on the ways that hormones interact with the developing immune system (Karmaus and Botezan, 2002).

There are a number of significant limitations in this study. First, IL-4 is only one cytokine in a vast network of cytokines, which is responsible for affecting biological changes. Future research will examine relationships between early microbial environments, IL-4, and additional cytokines such as interferon-gamma (IFN-γ), which is known to have antagonistic effects on IgE (Vercelli et al., 1990). Second, although our findings broadly support predictions made by the hygiene hypothesis, we did find some unexpected results, such as the significant positive association between the number of diarrheal episodes and IL-4, as well as the significant negative association between currently living in an urban household and IL-4.

We briefly discussed possible reasons that diarrheal episodes in infancy were associated with higher adult IL-4. However, more complicated explanations involving differential Th1 and Th2 development based on bacterial or viral infections may be applicable (Sergio, 1997). This could explain why we observed differential associations between IL-4 and diarrheal (possibly parasitic) and respiratory (likely viral) episodes of illness. Parasites may also explain our second unexpected finding that current urbanicity was negatively associated with IL-4. We had expected urbanicity to be associated with higher IL-4 based on evidence showing that atopic disease is more prevalent in urban rather than rural areas (Gale, 2002; Godfrey, 1975). However, research in Africa examining urban and rural communities found that despite the absence of asthma in rural areas, these groups had much higher IgE levels when compared with individuals in the urban area. These differences were most likely attributable to endemic parasitism (Merrett et al., 1976). These limitations and unexpected findings highlight the need for more extensive research on the links between early microbial environments, cytokines such as IL-4 and IFN-γ, and measures of both allergen-specific IgE and circulating IgE.

Our finding that microbial exposures during infancy predict concentrations of IL-4 more than 20 years later

builds on a growing body of literature demonstrating that early ecological conditions have long-term effects on human biology. Whether associations between early microbial environments and adult IL-4 can be linked to the production of IgE and the expression of atopic and allergic diseases in adulthood awaits future research. Regardless, these findings contribute to an emerging body of research in human ecological immunology (Blackwell et al., 2010; McDade, 2003a; Muehlenbein et al., 2010) and demonstrate the value of a population-based ecological approach to the study of human immune function.

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