Evaluation of airway reactivity and immune characteristics as risk factors for wheezing early in life

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Background: Childhood asthma is most often characterized by recurrent wheezing, airway hyperreactivity, and atopy; however, our understanding of these relationships from early in life remains unclear. Respiratory tract illnesses and atopic sensitization early in life might produce an interaction between innate and acquired immune responses, leading to airway inflammation and heightened airway reactivity.

Objective: We hypothesized that premorbid airway reactivity and immunologic characteristics of infants without prior episodes of wheezing would be associated with subsequent wheezing during a 1-year follow-up.

Methods: One hundred sixteen infants with chronic dermatitis were enrolled before episodes of wheezing. Airway reactivity, allergen-specific IgE levels, cytokine production by stimulated PBMCs, and percentages of dendritic cells were measured on entry, and airway reactivity was reassessed at the 1-year follow-up. Linear regression models were used to evaluate a predictor's effect on continuous outcomes.

Results: Milk sensitization, egg sensitization, or both were associated with heightened airway reactivity before wheezing and after the onset of wheezing; however, these factors were not associated with an increased risk of wheezing. There was an interaction between initial airway reactivity and wheezing as a determinant of airway reactivity at follow-up. In addition, cytokine production by stimulated PBMCs was a risk factor for wheezing, whereas increased percentages of conventional dendritic cells were protective against wheezing.

Conclusion: Our data in a selected cohort of infants support a model with multiple risk factors for subsequent wheezing that are independent of initial airway reactivity; however, the causative factors that produce wheezing very early in life might contribute to heightened airway reactivity. (J Allergy Clin Immunol 2010;126:483-8.)

Key words: *Infants, wheezing, airway reactivity, atopy, dermatitis, cytokines, dendritic cells, food allergy*

Episodes of wheezing early in life are primarily related to viral respiratory tract illnesses, which are important risk factors for childhood asthma; however, most infants and toddlers who wheeze do not have asthma.[1,2] Atopic sensitization early in life, which is most often to egg or milk, is also a risk factor for childhood asthma; however, not all atopic infants have asthma.[3] Infants and toddlers with atopy and recurrent wheeze early in life are at greater risk for childhood asthma than nonatopic subjects who wheeze.[4,5] In addition, reduced IFN-γ production from stimulated mononuclear cells obtained from infants before wheezing is a risk factor for subsequent wheezing.[6,7] This finding suggests that the infant's immune responsiveness to environmental stimuli, which is promoted by antigen-presenting cells, such as dendritic cells, is also an important determinant of wheezing and the development of asthma. Therefore investigators have proposed a hypothesis that requires 2 hits early in life for the development of childhood asthma: viral respiratory tract infections and atopic sensitization produce an interaction between innate and acquired immune responses, leading to airway inflammation and persistently altered airway function.[8] Childhood asthma is most often characterized by recurrent wheezing, airway hyperreactivity, and atopy; however, our understanding of the development of these relationships from early in life remains unclear.

We previously described that airway reactivity was heightened in infants with eczema (atopic and nonatopic based on the presence or absence of specific IgE); infants sensitized to egg, milk, or both; and those recruited before wheezing.[9] In our current 1-year follow-up, we hypothesized that the infant's premorbid immunologic characteristics (allergen-specific IgE levels, cytokine production by stimulated PBMCs, and percentages of dendritic cells in the peripheral blood), as well as airway reactivity, would be associated with episodes of wheezing during the 1-year follow-up. In addition, if major determinants of airway reactivity were present on entry to the study, airway reactivity would track longitudinally. Lastly, a 2-hit hypothesis suggests that wheezing would modify premorbid airway reactivity as a determinant of airway reactivity at the 1-year follow-up.

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Abbreviations used
cDC: Conventional dendritic cell
PC_{30}: Provocative concentration of methacholine to reduce forced expiratory flow at 75% expired volume by 30%
pDC: Plasmacytoid dendritic cell
PMA: Phorbol 12-myristate 13-acetate

METHODS

Subjects

The infants and toddlers with chronic dermatitis (n = 116) recruited for this study have previously been described.[9] Subjects were excluded for a history of prior wheezing, lower respiratory tract illness, treatment with asthma medications, or congenital heart disease. The institutional review board approved the study, and informed consent was obtained from parents.

Airway reactivity

After chloral hydrate sedation (50-100 mg/kg), airway reactivity was quantified based on the provocative concentration of methacholine to reduce forced expiratory flow at 75% expired volume by 30% (PC_{30}).[10]

Dermatitis

Severity was quantified by using SCORAD scores.[11]

Respiratory history

Maternal history of cigarette smoking during pregnancy (any vs none) and family history of asthma or allergy (parents or siblings) were obtained at initial and 1-year follow-up visits. Wheezy illnesses and medication use were updated by monthly telephone contact.

Immune characteristics

Atopic status. Total IgE and allergen-specific IgE levels (egg white, milk, wheat, cat, house dust mite, timothy grass, Bermuda grass, ragweed, *Alternaria* species, and cedar) were measured from blood samples (Immune Tech, Inc, Menlo Park, Calif). Subjects were characterized as atopic if there was any specific IgE level of greater than 0.35 IU/mL.

PBMCs. Techniques for isolation, culture, and stimulation (phorbol 12-myristate 13-acetate [PMA] plus ionomycin; egg or milk antigens), as well as measurement of cytokine production and staining for flow cytometric identification of cell populations, are summarized in this article's Online Repository at www.jacionline.org.

Statistical analysis

For association studies using regression models, PC_{30} values and dendritic cell variables were log transformed because of the right-skewed distribution. The ratio of T_H2/T_H1 cytokines was log transformed for normality. Analyses were adjusted for enrollment age, sex, race, and maternal smoking during pregnancy. PC_{30} values at entry into the study; sensitization to egg, milk, or both; and the number of wheezing episodes as predictors of PC_{30} values at the 1-year follow-up were evaluated by using linear regression models. A linear regression model including interaction between PC_{30} values at entry and wheezing episodes was fitted to evaluate the modification of wheezing episodes on the effect of PC_{30} values at entry on 1-year PC_{30} values. A Poisson regression model was used for each variable to evaluate dendritic cell numbers and cytokine production by PBMCs as predictors of the number of wheezing episodes during the 1-year follow-up. Forward stepwise regression was used to further select predictors among all predictors; the cutoff *P* value for inclusion was .15. Models for the number of wheezing episodes adjusting for interaction between smoking during pregnancy and each cytokine production variable were also fitted to evaluate the interaction effect. Cytokine production after egg or milk antigen stimulation by PBMCs was compared between the subjects with and without specific IgE to egg or milk antigen by using 2-sample *t* tests.

TABLE I. Demographics for subjects

	1-y Follow-up	Initial (dropped out)	*P* value*
No. of subjects	98	18	
Sex (% male)	49 (50%)	7 (39%)	.386
Race (% white)	48 (49%)	5 (28%)	.097
Family history of asthma/allergy† (% yes)	82 (85%)	11 (61%)	.120
Maternal smoking during pregnancy (% yes)	8 (8%)	5 (28%)	.030

*The *P* values are for comparison of baseline characteristics between the subjects who dropped out and those who did not by using 2-sample *t* tests for continuous variables and χ^2 tests for binary variables. Smoking during pregnancy was different between the group of drop outs and the group who did not drop out.
†Family history was not available for 1 subject.

RESULTS

Subjects

Of 116 subjects recruited at a median age of 10.7 months (range, 2.6-19.1 months), 98 remained at the 1-year follow-up (Table I). The group of infants who dropped out had a higher percentage with a maternal history of smoking compared with the group who completed the 1-year follow-up evaluation. At follow-up, subjects were evenly divided by sex and race. In addition, 77% of the subjects had an immediate family history of asthma or allergy, whereas 11% had a history of maternal smoking during pregnancy. Atopic subjects had higher total IgE levels (log) at baseline (1.08 vs 0.68, $P < .006$) and follow-up (1.58 vs 1.04, $P < .001$).

There were missing data for airway reactivity for 8 subjects who did not sleep for the measurement at entry to the study and 2 subjects at follow-up. Subjects did not undergo bronchial challenge after baseline forced expiratory flow at 75% of expired volume of less than 2 SDs below the predicted value (n = 11 at initial evaluation and n = 1 at follow-up). These 11 subjects did not differ from the remaining subjects with respect to any other measurements other than that there were fewer male subjects (18% vs 51%, $P = .04$). Venipuncture was not successful for 3 subjects.

Frequency of wheezing during 1-year follow-up

Forty-eight percent of infants had no reported wheezing, 31% had 1 or 2 episodes, and 21% had more than 3 episodes during the 1-year follow-up. Among subjects with reported wheezing, 75% had wheeze only with colds, whereas 25% had wheezing with and without colds.

Predictors of wheezing

Table II and Fig 1 summarize the predictors of wheezing that were evaluated. An increased odds ratio for risk of wheezing was associated with maternal smoking during pregnancy (1.6, $P < .034$); there was a very small decreased risk with increased age at enrollment (0.96, $P < .019$) and a tendency for a decreased risk of wheezing for female subjects (0.76, $P = .090$), whereas race was not significant (1.18, $P = .296$).

Airway reactivity. Among the subjects with airway reactivity assessed on entry to the study (n = 89), the median PC_{30} value was 0.418 mg/mL (range, 0.046-10.000 mg/mL). A lower PC_{30} value (greater reactivity) was not a significant risk factor for an increased number of wheezing episodes ($P > .12$).

Allergen-specific IgE. Atopy defined as specific IgE to egg antigens, milk antigens, or both or as specific IgE to any of the 10 antigens was not associated with an increased risk of wheezing.

Cytokine production. We observed that increased ratios of IL-4/IFN-γ, IL-5/IFN-γ, and IL-10/IFN-γ were associated with an increased risk for wheezing (Table III and Fig 1). In addition to maternal smoking being a significant predictor of increased risk of wheezing (odds ratio, 1.6; $P < .041$) independent of cytokines, there was a significant interaction between maternal smoking during pregnancy and cytokine production as a risk for increased episodes of wheezing. Increasing cytokine production (IL-4/IFN-γ and IL-5/IFN-γ) was associated with increasing numbers of wheezing episodes ($P < .027$) for subjects with maternal smoking during pregnancy but not for subjects without maternal smoking during pregnancy.

There were no significant associations between ratios of cytokines produced by PBMCs after PMA/ionomycin and atopic status defined as specific IgE to egg or milk antigens ($P > .6$, Fig 1). Despite the lack of an association of polyclonal cytokine production with specific IgE levels, there was an associated allergen-specific T-cell response. In a limited number of subjects with an adequate number of PBMCs available, we stimulated PBMCs from subjects who had positive results to eggs (n = 8) or milk protein (n = 9) and similar numbers of subjects who had negative results to all antigens. Production of IL-4, IL-5, IL-10, and IL-13, but not IFN-γ, by stimulated PBMCs was significantly greater for subjects with IgE specific to egg or milk compared with that seen in subjects with negative results for specific IgE to all 10 antigens (data not shown). There were no significant differences in demographics or total serum IgE levels among subjects with and without availability of additional PBMCs. These findings confirm the development of an allergen-specific T_H2 response.

Dendritic cells. There was a significant inverse relationship between the percentages of conventional dendritic cells (cDCs) in peripheral blood on entry into the study and the subsequent number of episodes of wheezing; greater percentages of dendritic cells were associated with fewer episodes of wheezing (Table III and Fig 1). The percentages of cDCs were not associated with cytokine production. There were no significant associations between T_H2 cells, regulatory T cells, or natural killer T cells and wheezing. When all 5 covariates in Table III were included in a stepwise model as predictors of the number of episodes of wheezing, cDCs remained significant ($P < .001$) and IL-10/IFN-γ approached significance ($P < .061$).

Predictors of airway reactivity at 1-year follow-up

Predictors of airway reactivity at the 1-year follow-up are shown in Table IV.

Allergen-specific IgE. Subjects with specific IgE to egg antigens, milk antigens, or both on entry into the study had lower PC_{30} values on entry into the study, as well as at the 1-year follow-up, compared with subjects with negative results for milk and egg (Table V). In addition, egg/milk sensitization at follow-up was still associated with heightened reactivity at follow-up (Table V and Fig 1). These relationships were not present when atopy was defined as specific IgE to any of the 10 antigens.

TABLE II. Predictors of wheezing

Age, race, sex, and maternal smoking during pregnancy
Airway reactivity on entry to the study
Specific IgE
Cytokine production by stimulated PBMCs
Dendritic cells

Airway reactivity on entry into the study. Airway reactivity on entry into the study was highly correlated with airway reactivity at the 1-year follow-up (n = 82); infants with lower PC_{30} values at the initial evaluation were more likely to have lower PC_{30} values at follow-up (Table V and Fig 1). There was also a significant relationship between episodes of wheezing and PC_{30} values at follow-up; the greater the number of episodes of wheezing, the lower the PC_{30} value. When all 4 covariates in Table V were included in a stepwise model as predictors of PC_{30} values at follow-up, PC_{30} values on entry into the study ($P < .001$) and egg/milk sensitization at follow-up ($P < .012$) remained significant covariates, adjusting for age, sex, race, and maternal smoking during pregnancy. When initial airway reactivity and subsequent episodes of wheezing were included in the model as predictors of airway reactivity at follow-up, adjusting for age at entry into the study, sex, race, and maternal smoking, there was a significant interaction term between initial airway reactivity and episodes of wheezing ($P < .006$). Among infants with lower PC_{30} values on entry into the study, those with more than 3 episodes of wheezing maintained lower PC_{30} values at follow-up; those infants with fewer than 3 episodes of wheezing had higher PC_{30} values at follow-up.

Cytokine production. Cytokine production was not associated with PC_{30} values on entry into the study; however, increased IL-10/IFN-γ levels were associated with decreased PC_{30} values at follow-up ($P < .037$).

Dendritic cells. Percentages of cDCs were not associated with PC_{30} values.

DISCUSSION

Our study has demonstrated that increased ratios of T_H2/T_H1 cytokine production by stimulated PBMCs before wheezing was a risk factor for subsequent wheezing, whereas increased dendritic cells in the blood before wheezing were protective against subsequent wheezing. Although allergen-specific IgE levels and airway reactivity on entry into the study were not associated with an increased risk for subsequent wheezing, specific IgE levels to egg or milk were not only associated with heightened airway reactivity before episodes of wheezing, but also this relationship persisted even after the onset of wheezing early in life. We also found that heightened airway reactivity before wheezing, subsequent episodes of wheezing, and an interaction between these parameters were associated with heightened airway reactivity at the 1-year follow-up. These later findings suggest that airway reactivity might be modified by causative factors contributing to wheezing early in life. Cumulatively, our data support a model wherein there are multiple risk factors for subsequent wheezing that are independent of initial airway reactivity; however, wheezing very early in life might contribute to the persistence of heightened premorbid airway reactivity.

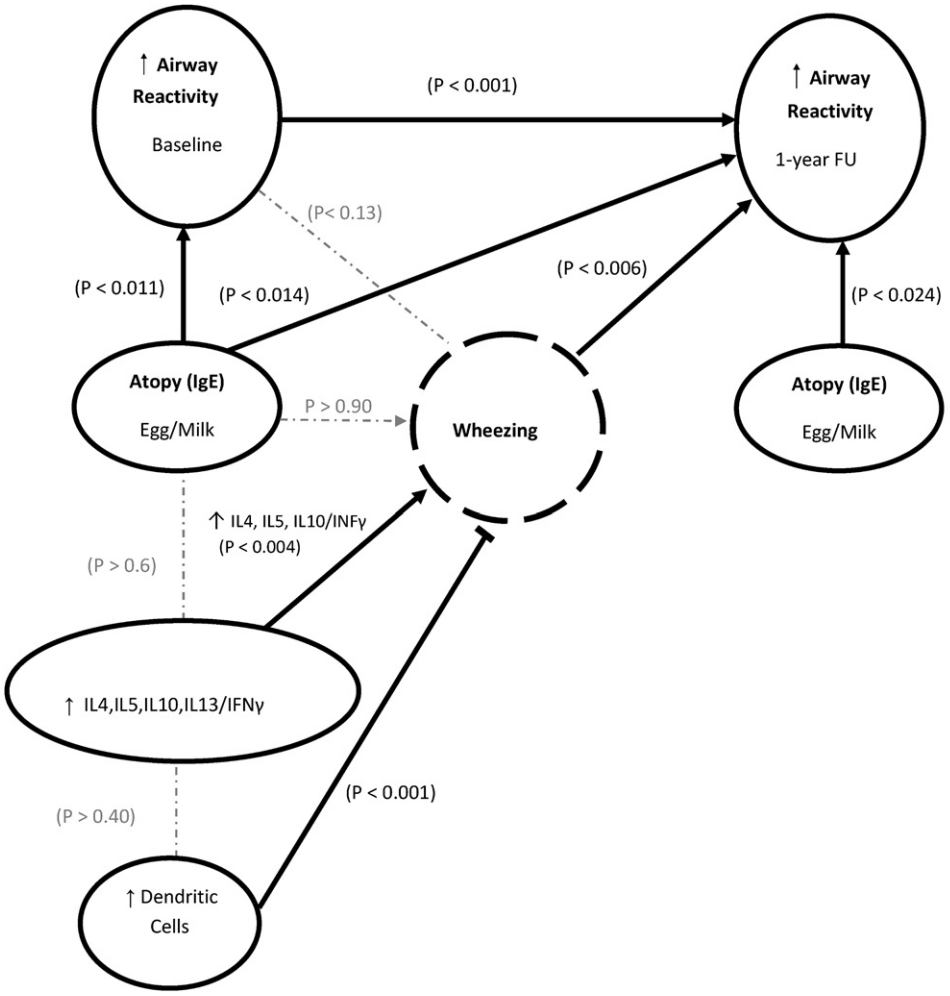


FIG 1. Illustration summarizing associations of airway reactivity, immune characteristics, and wheezing. →, Significant positive association; ⊣, significant negative association; – · –, no significant association. *FU*, Follow-up.

TABLE III. Cytokine production by PBMCs and dendritic cells at entry to the study as predictors of wheezing

Outcome	Covariate	Rate ratio	*P* value
Episodes of wheezing	IL-4/IFN-γ ln	1.33 (1.11-1.6)	.002
	IL-5/IFN-γ ln	1.33 (1.1-1.62)	.004
	IL-10/IFN-γ ln	1.35 (1.14-1.59)	<.001
	IL-13/IFN-γ ln	1.11 (0.93-1.34)	.258
	cDC ln	0.64 (0.52-0.79)	<.001

Cytokines and cDCs are natural log transformed *(ln)*. At each row, a Poisson regression model was fit and adjusted for age at entry to the study, sex, race, and maternal smoking during pregnancy.

TABLE IV. Predictors of airway reactivity at the 1-year follow-up

Age, race, sex, and maternal smoking during pregnancy
Specific IgE
Airway reactivity on entry to the study
Episodes of wheezing
Cytokine production by stimulated PBMCs
Dendritic cells

We did find that the ratio of T_H2/IFN-γ cytokine production from stimulated PBMCs obtained on entry into the study was a significant risk factor for subsequent wheezing at the 1-year follow-up. The ratio was used to assess the balance between these components of immune response. In the Tucson cohort study investigators have reported that lower levels of IFN-γ were associated with an increased risk for wheezing early in life,[6,7] and we found similar results in our data when analyzed for absolute amounts of IFN-γ. However, we found that by using absolute values for T_H2 cytokines, increased levels were associated with decreased, rather than increased, wheezing. This suggests that the risk of wheezing might be associated with an overall decreased cytokine response by stimulated PBMCs (T_H2 and T_H1). Despite this, an increased T_H2/T_H1 cytokine ratio is still associated with an increased risk for wheezing. This might reflect that pathology depends not only on the absolute amount of cytokines in the system but also on the balance of signals provided by cytokines that have opposing effects on cells. Zhang et al[12] recently reported that an increased IL-10/IL-5 ratio after PHA stimulation of cord blood mononuclear cells was associated with a decreased risk of wheezing during childhood. In our study we found that increased IL-10/IL-5 production by PBMCs after

TABLE V. Airway reactivity at the 1-year follow-up

Variable	Parameter	Estimate	SE	*P* value
PC_{30} ln at 1-y follow-up	PC_{30} ln at entry to the study	0.578	0.097	<.001
	No. of wheezing episodes	−0.144	0.051	.006
	Egg or milk positive response at entry to the study	−0.601	0.239	.014
	Egg or milk positive response at 1-y follow-up	−0.541	0.234	.024

PC_{30} values were natural log transformed *(ln)*. At each row, a linear regression model was fit and adjusted for age at entry to the study, sex, race, and maternal smoking during pregnancy.

PMA/ionomycin stimulation was associated with an increased risk of wheezing. The results of these studies might differ because of populations of subjects evaluated, the source of the mononuclear cell stimulated, the stimulation, and the length of follow-up.

A previous report identified a protective effect of plasmacytoid dendritic cell (pDC) numbers on subsequent wheezing episodes and the development of asthma,[13] although they did not observe an association with cDCs (termed "mDCs" in their study). We observed a similar protective effect of pDCs on subsequent wheezing in our study (data not shown), but we also observed that increased numbers of cDCs in peripheral blood obtained from infants before wheezing were protective against subsequent episodes of wheezing. One likely explanation for this difference is in how the dendritic cell subsets were defined. The similar results for pDCs, which Upham et al[13] defined as CD11c intermediate and CD23 positive and we defined as blood dendritic cells antigen (BDCA-2) positive but negative for CD19 and CD14, suggest that these populations overlap. However, the distinct results for cDCs, defined by Upham et al as CD11c and HLA-DR positive and defined by us as BDCA-1 positive but negative for CD19 and CD14, suggest that the precise populations of cells identified might be different. Because many early episodes of wheezing are triggered by viral infections, Upham et al proposed that increased pDC numbers might promote better clearing of viral infections through production of type I interferons. Similarly, increased numbers of cDCs might promote increased antiviral immune responses. Indeed, the percentages of cDCs, as well as the absolute amount of IFN-γ produced in culture, were inversely correlated with subsequent wheezing, suggesting a potential pathway. Thus the negative effect of cDCs on wheezing might be indirect.

We found that airway reactivity on entry into the study before any episodes of wheezing was highly correlated with airway reactivity at the 1-year follow-up. This longitudinal finding has not previously been reported in this age group. In addition, infants and toddlers with specific IgE to egg, milk, or both at follow-up had heightened airway reactivity at follow-up compared with that seen in subjects with negative results for all IgE-specific antigens evaluated. This current finding on the relationship between sensitization to egg, milk, or both early in life and airway reactivity is consistent with our previous report relating egg/milk sensitizations to airway reactivity on entry into the study and indicates that this relationship is not transient.[9] Illi et al[3] reported that food sensitization before 2 years of age is a risk factor for asthma as an older child, whereas inhalant sensitization before 2 years of age without concurrent food sensitization does not increase the risk for asthma.[3] There are multiple determinants of airway reactivity; however, our findings suggest that significant components of airway reactivity are present very early in life. The persistence of a relationship between egg/milk sensitization and airway reactivity might identify subjects who will maintain heightened airway reactivity and have asthma as an older child. The absence of an association between sensitization to egg or milk and wheezing early in life might reflect that wheezing at this young age is not primarily related to atopy or airway reactivity.

We had previously reported that infants and toddlers had heightened airway reactivity several months after a wheezy respiratory tract illness, which was still present 8 months after the acute illness.[14] Similarly, Saga et al[15] reported that heightened airway reactivity among wheezy infants was associated with having asthma as an older child. However, in the absence of a premorbid assessment of airway reactivity, it is difficult to interpret the relationships between airway reactivity and wheezing. In our current study we did not find that premorbid airway reactivity was a risk factor for subsequent wheezing. This finding differs from the results of Clarke et al,[16] who assessed the relationship between airway reactivity at 1 month of age and the risk of wheezing during the 1-year follow-up. In their population of neonates selected for family asthma, these investigators did not find an association between airway reactivity and a single wheezy illness; however, infants with more than 3 episodes of wheeze had heightened premorbid airway reactivity compared with subjects with less than 2 episodes of wheeze. The results of these 2 studies might have differed because of the selected populations. Our subjects were older (10 months vs 1 month) and selected based on the criterion of eczema rather than a family history of asthma; therefore we might have excluded subjects with heightened airway reactivity who wheezed at a younger age.

In the current study we measured both premorbid airway reactivity and that at the 1-year follow-up; therefore we were also able to evaluate the relationship that premorbid airway reactivity and subsequent episodes of wheezing have with airway reactivity at follow-up. Although premorbid airway reactivity was not directly associated with risk of wheezing, the number of episodes of wheezing was associated with airway reactivity at the 1-year follow-up; subjects with more episodes of wheezing had a lower PC_{30} value or heightened airway reactivity. There was also a significant interaction between premorbid airway reactivity and wheezing as predictors of airway reactivity at follow-up. Among infants with heightened premorbid airway reactivity, those with an increased number of episodes of wheezing were more likely to maintain heightened airway reactivity at follow-up. The only other longitudinal study of airway reactivity with premorbid measurements during infancy is from the Perth cohort.[17] Our findings in a selected cohort are consistent with their recently reported long-term follow-up of an unselected cohort showing that heightened airway reactivity during infancy was associated with heightened airway reactivity in childhood for subjects with a lower respiratory tract illness before 6 months of age. Although these investigators have not reported the relationship between wheezy illness and airway reactivity early in life, they have reported that heightened airway reactivity at 12 months of age was more

consistently associated with asthma outcomes as an older child than airway reactivity at 1 or 6 months of age. Their long-term and our short-term follow-up are consistent with the hypothesis that respiratory tract illnesses early in life contribute to the persistence of heightened airway reactivity.

There are several limitations to our study. We enrolled only infants with chronic dermatitis (atopic and nonatopic); there were no subjects without any dermatitis. There is support for 2 variants of eczema[18]; subjects with nonatopic eczema have a lower risk of asthma than those with atopic eczema.[19,20] In addition, because our subjects were not a birth cohort but were recruited with eczema, those who were older on entry had gone slightly longer without wheezing, which might provide a small selection bias because the prevalence of wheezing decreases with increasing age. This could contribute to our finding that increasing age on entry into the study was associated with a very small but statistically significant decrease in the risk of subsequent wheezing. We did not specifically evaluate whether wheezing illnesses were associated with viral infections; however, most families reported that wheezy illnesses occurred with colds, which is consistent with the importance of viral agents, such as respiratory syncytial virus and rhinovirus, as causative factors for wheeze in this age group.[2] Lastly, episodes of wheezing were determined by parents and not a physician; however, information was obtained from monthly telephone contact, which should minimize errors related to recall.

In summary, in our selected cohort of infants, we found a relationship between milk/egg sensitization and heightened airway reactivity that begins early in life before episodes of wheezing and persists after the onset of wheezing; however, these 2 factors were not associated with an increased risk of wheezing. In addition, we found an interaction between premorbid airway reactivity and episodes of wheezing, which suggests that airway reactivity at follow-up might be modified by factors contributing to wheezing early in life. These factors might be related to T_H2/T_H1 cytokine production by stimulated PBMCs, which was a risk factor for wheezing, as well as increased dendritic cells in the blood, which was protective for wheezing. Our data support a model with multiple risk factors for subsequent wheezing that are independent of initial airway reactivity; however, the causative factors that produce wheezing very early in life might contribute to the persistence of heightened airway reactivity.

Clinical implications: Airway reactivity, atopy, cytokine production by stimulated PBMCs, and the number of dendritic cells contribute to the risk for recurrent wheezing and airway hyperreactivity early in life.

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METHODS

Isolation and culture of peripheral blood mononuclear cells (PBMC)

Four mL venous blood samples were separated on a gradient of Ficoll-paque PLUS (GE Healthcare, Sweden). Cells were counted with trypan blue and stored in DMSO-freezing medium (Bioveris). PBMC were stimulated with Phorbol myristilacetate (PMA, 10 ng/mL) + ionomycin (IONO, 500 ng/mL) in complete RPMI-1640 (Invitrogen, Rockville, Md) for 72 hours or stimulated with egg and milk antigens (Invivogen) for 24 hours at 37°C in a humidified 5% CO_2 incubator. PBMC Supernatants were harvested and stored at -80°C for cytokine analysis.

Cytokine multiplex analysis

IL-4, IL-5, IL-10, IL-13 and IFN-γ were measured using the Multiplex Bead Immunoassays as per manufacturer's protocol (Millipore, Saint Charles, Mo) using a Luminex 200 instrument (Luminex Corporation, Austin, Tex). Cytokine concentrations were calculated using Bio-Plex Manager 2.3 software with a five parameter curve-fitting algorithm applied for standard curve calculations. The minimal cytokine detection limit is 3.2 pg/mL; the frequency of non-detection for all five cytokines was 0.

Dendritic Cell Staining

Conventional dendritic cells (cDC) were isolated and stained using a blood dendritic cell enumeration kit following manufacturer instructions (Miltenyi Biotec). Samples were analyzed by FACScalibur cytometer (Becton Dickinson, San Jose, Calif). For dendritic cells, the minimally detectable limit is 0.005%; the frequency of non-detection was 0.

Th2, Treg, and NKT staining

PBMCs were stained with antibodies against human-CD4, CRTH2, CD25, NKT (BD PharMingen, San Diego, Calif), and Foxp3 (e-Bioscience, San Diego, Calif) directly conjugated to FITC, PE, PerCP or APC. Cells were fixed and analyzed in a FACScalibur cytometer.