Hyper-responsive T-cell cytokine profile in association with development of early childhood wheeze but not eczema at 2 years

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Summary

Background: **Eczema is a known risk factor for the development of wheeze in childhood. Cord blood T-cell cytokine responses have been shown to be associated with the development of both early childhood eczema and wheeze. Our objective is to study and compare the influence of intrinsic T-cell cytokine responses on the development of wheezing and eczema in the first 2 years of life in a birth cohort of at risk (first degree family with atopic disease) infants.**

Methods: **Cord blood samples were collected from 195 eligible subjects of a birth cohort of 253 subjects. The subjects studied were those who developed either wheezing (n = 34) or eczema (n = 29) in the first 2 years of life, and 65 healthy infants served as control. Cytokines from phytohaemagglutinin stimulated mononuclear cells were analyzed using multiplex cytokine assays and the cytokine profiles in the 3 groups were compared.**

Results: **Most of the subjects were non-atopic with only 3/34 (9%) wheeze and 9/29 (31%) eczema subjects sensitized to the common dietary or inhalant allergens. After adjustment for potential risk factors, wheeze, but not eczema subjects, presented with hyper-responsive cytokine profiles with increased production of T-cell cytokines IL-2 and IL-5. IL-5 was the strongest risk factor associated to the development of wheeze at 2 years of age (OR, 35; 95% CI, 5.0 – 246.7).**

Conclusion: **Cord blood cytokine responses in early onset wheeze and eczema are distinctly different. This suggests that the tendency to develop early onset wheeze may be influenced by preexisting immune factors independent to those for eczema.** ***(Asian Pac J Allergy Immunol 2014;32:84-92)***

Key words: *Cord blood mononuclear cells, phytohaemagglutinin, early onset wheeze, infant eczema, cytokines*

Introduction

Wheezing in early childhood is a heterogeneous condition with a long term prognosis that varies from a transient condition with complete recovery[1] or to recurrent wheeze or asthma later on in life.[2] Eczema in the first year of life is an independent risk factor for wheezing, and chronic asthma.[3]

Genetic or environmental factors contribute to the development of infant wheeze,[4] and viral respiratory tract illnesses caused by respiratory syncytial virus (RSV) and rhinovirus (RV) in early life have also been implicated as contributors to this outcome by causing lower airway infections and inducing wheezing in young children. Respiratory viruses were strongly associated with wheezing and were detected in at least 80% of wheezing infants with the predominant pathogens being RSV in infants younger than 2 years of age and the rhinovirus in subjects older than 2 years of age.[5] More importantly, it was reported that wheezing with RSV, RV or both at the third year of life was associated with increased asthma risk at age 6 years from a high-risk birth cohort.[6]

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The immune response, in particular T cell responses are known to play a role in the development of viral-induced wheeze. Tregoning et al. have reported that $CD8^+$ T cells are associated with wheeze exacerbation and depletion of $CD8^+$ T cells during primary RSV viral infection of neonatal BALB/c mice inhibited disease progression.[7] Young children with RV-triggered wheeze had higher serum T-cell cytokine IL-10, IL-2, IFN-γ and IL-5 concentrations compared to healthy non-allergic individuals.[8] Furthermore, both $CD4^+$ and $CD8^+$ T lymphocyte produced a higher percentage of IL-4, a type-2 cytokine following an RSV infection in infants with wheeze compared to healthy controls.[9]

Pre-existing alterations of the immune system, such as cytokine dysregulation and Th1/Th2 imbalance detected at birth, have also been shown to influence host susceptibility to the development of atopy, wheeze and eczema.[10,11,12] In this study, we have undertaken to evaluate and compare cord blood mononuclear cell (CBMCs) T-cell cytokine responses in a clinically well-defined birth cohort of at-risk infants (first degree relative with allergic disease) with distinct clinical outcomes of either wheeze or eczema at the age of 2 years.

Methods

Study population

We analyzed cord blood samples from 128 newborns originally recruited for a probiotic intervention trial (ClinicalTrials.gov Identifier: NCT00318695). This group represents a subset of 253 infants enrolled at the National University Hospital of Singapore that were required to have at least one parent with allergic rhinitis, eczema or asthma. The children in the cohort have been followed up prospectively. The primary clinical outcome measure in this study was the incidence of eczema, and the secondary outcome measures were wheeze, allergic rhinitis and allergen sensitization. Infants were evaluated by a pediatrician at 1, 3, 6, 12 and 24 months of age. Eczema was defined as a pruritic rash over the face and/or extensors with a chronic relapsing course, as described by Hanifin and Rajka and modified by Seymour et al.[13] for infants while the SCORAD (SCORing Atopic Dermatitis) index was used to objectively score the severity of atopic dermatitis. Frequent wheeze/asthma was diagnosed if the child had three episodes of nocturnal cough with sleep disturbances or wheezing symptoms, separated by at least seven days, in a setting where asthma was likely and conditions other than allergy have been excluded. Serum was collected at 12 months of age and stored at -70°C before assayed for total IgE using the fluoroenzymeimmunoassay method (UniCAPs Phadiatop, Pharmacia Diagnostics, Uppsala, Sweden) with a detection limit of 0.35 kU/L. Skin prick tests were performed at 12 months of age using common food and inhalant allergens: milk, egg yolk, egg white (Alyostal, Stallergenes Laboratoires, Antony Cedex, France), dust mite allergens – *Dermatophagoides pteronyssinus* (Greer Laboratories, Lenoir, NC, USA) and *Blomia tropicalis* manufactured in-house.[14] Written informed consents were obtained from all families. The study was approved by the National University Hospital's ethics review committee (DSRB Ref Code: B/00/322).

Demographic characteristics

In this study, subjects were divided into 3 groups according to their clinical outcomes and subjects from both the placebo and probiotic group were included in this study.

Group 1: infants who developed wheeze (n = 34), Group 2: infants who developed eczema (n = 29), and Group 3: healthy control infants (n = 65) with no clinical manifestations of eczema, wheeze or any atopic disorder, and were not allergen sensitized to either dietary or inhalant allergens. There were only 3 (9%) subjects from the wheeze group and 9 (31%) subjects from the eczema group and who were sensitized to either common dietary or inhalant allergens at 24 months of age. In the Group 1 wheeze subjects, the percentages of infants who developed wheeze below 6 months, between 7 to 12 months and between 13 to 24 months of age were 24%, 44% and 32%, respectively. There were 16 subjects with a single episode, 14 subjects with 2-3 episodes and only 4 with more than 3 episodes of wheeze. The majority of the group 2 eczema subjects developed eczema by the age of 6 months (n = 22, 76%). The mean SCORAD index of the infants with eczema at 24 months was 16.3. In order to evaluate wheeze and eczema outcomes separately, subjects with both eczema and wheeze (n = 7) were excluded from the study. In addition, another 43 subjects were excluded and these included those allergen sensitized subjects without any clinical manifestations (n = 32), and subjects with only transient eczema in early infancy (n = 11).

Stimulation of cord blood mononuclear cells with phytohaemaglutinin

The umbilical cord blood collected was processed within 12-24 hours and stored in liquid nitrogen (-150°C) until further analysis was carried out. The frozen CBMCs were thawed quickly in a 37°C water bath; the viability of the cells was 70% (median, ranged from 67.5% to 72.5%) as determined by Trypan blue staining. The viability of our cells were comparable to cell viabilities in studies by Chen et al. (65-85% viability)[15] and Hayakawa et al. (75% ± 11% viability).[16] CBMCs were plated in 96-well round-bottom cell-culture plates in triplicates at 2×10^5 viable cells/well in 200 µl of AIM-V medium (Gibco Life Technology, Grand Island, NY, USA). Phytohaemagglutinin (PHA) (Sigma-Aldrich, St Louis, MO, USA) stimulation was done at concentrations ranging from 0 to 5 µg/mL (0, 0.1, 0.2, 0.5, 1, 2.5 and 5 µg/mL) and culture supernatants were collected at 24 and 48 hours after incubation at 37°C with 5% CO_2 and stored at -80˚C. For the cohort study, the cytokine profile from the 48-hour culture supernatants from 5 µg/mL of PHA stimulation was analyzed with clinical outcomes.

Cytokine detection

Cytokine concentrations in culture supernatants, IL-2, IFN-γ, IL-5 and IL-13 were determined using the Cytometric Bead Array human cytokine Flex Sets and measured by the BD FACSArray™ Bioanalyzer (BD Biosciences, San Jose, CA, USA) according to manufacturer's instruction. The detection limit for all cytokines was 10 pg/mL. The presented concentrations were those evoked by the PHA stimulation after subtraction of responses from unstimulated cultures from each individual.

Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 for windows (SPSS, Inc. Chicago IL, USA). Differences in the demographic data were tested for significance using the Mann-Whitney *U* test for non-normally distributed numerical data and the Pearson chi-squared test was used for categorical data. The Mann-Whitney *U* test was performed to assess the differences in cytokine responses between the 3 groups, wheeze, eczema and controls. The *p* value was adjusted based on the Bonferroni correction to account for multiple comparisons of the 3 groups. Significant differences in the demographic data between groups by univariate analysis were included in multivariate logistic regression analysis to adjust for these potential confounders in the cytokine responses. Values of $p \leq 0.05$ were considered statistically significant. Boxplot figures were drawn using the GraphPad Prism 5 software (La Jolla, CA, USA).

Results

Kinetics and dosage responses of phytohaemagglutinin stimulated T cell cytokine production from cord blood mononuclear cells

IL-2, IFN-γ, IL-5 and IL-13 produced from CBMCs stimulated with various concentrations of PHA were first assessed to determine the optimal PHA dosage and time point to collect supernatants for the following experiments in this study. All the cytokine levels were significantly higher at a time point of 48 hours, as compared to 24 hours at the PHA concentration of 5 µg/mL (Figure 1). The

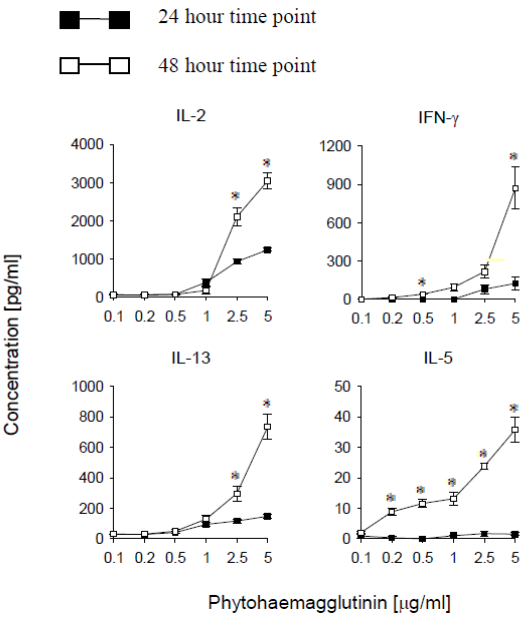

Figure 1. Kinetic and dosage response curves for phytohaemagglutinin (PHA) stimulated cord blood mononuclear cells (CBMCs). CBMCs were plated in 96-well round-bottom cell-culture plates in triplicates at 2×10^5 viable cells/well in 200 µl of AIM-V medium and PHA stimulation was done at concentrations ranging from 0 to 5 µg/mL (0, 0.1, 0.2, 0.5, 1, 2.5 and 5 µg/mL). Culture supernatants were collected at 24 and 48 hours and cytokines IL-2, IFN-γ, IL-5 and IL-13 were measured. The open squares represent the 48 hour time point and the closed squares represent the 24 hour time point. Data are expressed as mean ± standard deviation of triplicate measurements *: $p < 0.05$.

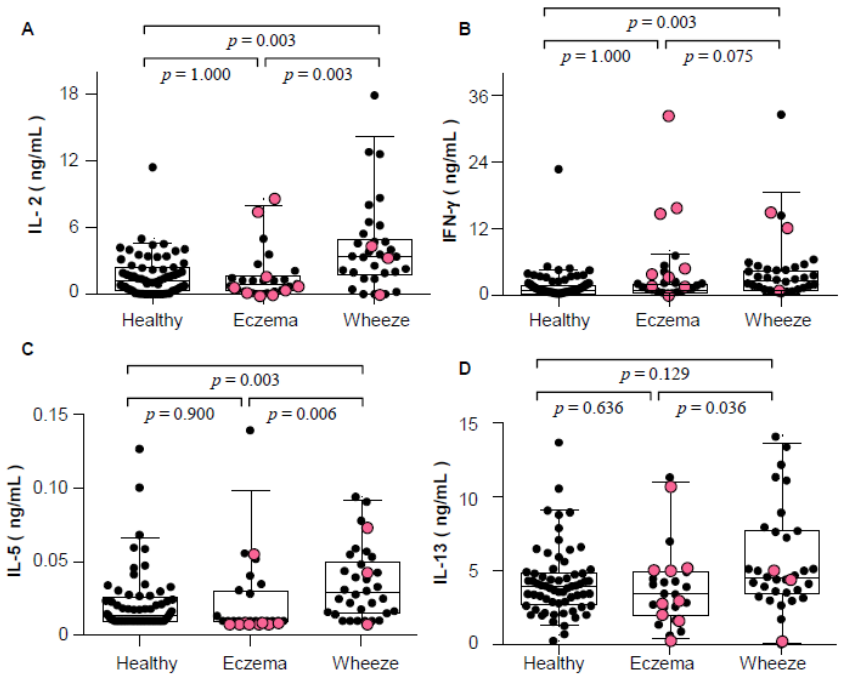

Figure 2. A- D , CBMC cytokine profile of healthy (n = 65), eczema (n = 29) and wheeze (n = 34) subjects in response to 5 μg/mL PHA. The black circles represent non-allergen sensitized subjects, while the pink circles represent allergen sensitized subjects. The band in the middle of the boxplot represents the median value, and the ends of the whiskers represent the 5th and 95th percentile values of the data.

cytokine readouts in Figure 1 at 48 hours show that a PHA concentration of 5 μg/mL induced the highest levels of cytokine responses for all cytokines measured in this cohort. In summary, a PHA concentration of 5 μg/mL and a time point of 48 hours for supernatant collection were the optimal conditions for use in our study which used an AIM-V serum free culture condition for CBMCs.

T-cell cytokine responses from phytohaemagglutinin *stimulated cord blood mononuclear cells*

The PHA stimulated CBMCs cytokine profiles are shown in Figure 2, expressed as medians and interquartile ranges, with *p* values adjusted using the Bonferroni method. A hyper- responsive cytokine profile was seen in the wheeze subjects versus the healthy controls, with increased naive T-cell cytokines: IL-2; 3.38 ng/mL (1.78 - 4.91) vs 1.23 ng/mL (0.29 - 2.40), IFN-γ; 2.71 (0.96 - 4.28) vs 0.75 ng/mL (0.33 - 1.86), and IL-5; 0.03 (0.02 - 0.05) vs 0.01 ng/mL (0.01 - 0.03) with $p = 0.003$ respectively (Figure 2). No significant differences were seen between the cytokines measured in the control group and the eczema group. Similar to the comparison with healthy controls, the wheeze subjects also had higher CBMCs T-cell cytokine profiles compared to eczema subjects (IL-2, IL-5, and IFN-γ, $p < 0.05$).

To further refine the clinical phenotypes evaluated, the data were also analyzed for the group with non-atopic wheeze (n = 31/34) and non-atopic eczema (n = 20/29) (that is absence of allergen sensitization at 24 months). The multi comparison of the healthy, eczema and wheeze cytokine profiles in this subset did not change and maintained a similar trend for all the PHA cytokine profiles. Figure 2 shows the even spread of the scatter of individual data points of the sensitized (pink circles) versus the non-sensitized (black circles) subjects throughout the range cytokine concentrations detected.

Logistic regression analysis

The effects of confounding factors arising from demographic and clinical factors on individual cytokine profiles were evaluated. Based on factors that were statistically different between groups in the univariate analysis (Table 1), the comparisons of cytokine profiles were adjusted for the following variables: mode of delivery, maternal asthma, birth order, birth weight and birth length for the wheeze and control group; and gestational age and paternal eczema for eczema and control groups. The subjects

Table 1. Clinical demographics of subjects in the cord blood mononuclear cell (CBMC) cytokine study.

	Healthy (n=65)	Eczema (n=29)	Wheeze (n=34)	Healthy vs. Eczema P value	Healthy vs. Wheeze P value
Gestational age in weeks median (IQR)	38.7 (1.6)	39.6 (1.7)	39.3 (1.9)	0.021	0.088
Mode of delivery, no.(%) Lower segment caesarean section	20 (31)	6 (21)	4 (12)	0.313	**0.036**
Family history 1st degree relative (%) Maternal asthma	8 (12)	5 (17)	12 (35)	0.522	**0.007**
Maternal eczema	12 (18)	4 (14)	4 (12)	0.578	0.39
Maternal allergic rhinitis	23 (38)	16 (36)	16 (47)	0.072	0.357
Paternal asthma	12 (18)	4 (14)	5 (15)	0.578	0.638
Paternal eczema	5 (8)	7 (24)	2 (6)	0.027	0.739
Paternal allergic rhinitis	19 (32)	9 (31)	8 (24)	0.86	0.545
Sibling asthma	6 (8)	5 (17)	8 (24)	0.264	0.053
Sibling eczema	7 (12)	5 (17)	6 (18)	0.385	0.336
Sibling allergic rhinitis	7 (11)	4 (14)	6 (18)	0.674	0.336
Birth order 1 2 >3	27 (42) 13 (20) 25 (38)	14 (45) 6 (20) 9 (35)	7 (23) 14 (45) 13 (32)	0.771	**0.038**
Birth Height in median (IQR)	49 (3)	50 (2.0)	50 (4)	0.076	**0.003**
Birth weight in median (IQR)	3.0 (0.5)	3.1 (0.5)	3.3 (0.5)	0.147	**0.01**

Statistically significant differences ($p < 0.05$) are highlighted in bold.

with eczema had higher gestational weight compared to healthy subjects ($p = 0.021$) and had a higher percentage of fathers with eczema ($p = 0.027$). More of the wheeze subjects were delivery vaginally compared to the healthy subjects ($p = 0.036$), were taller ($p = 0.003$) and heavier ($p = 0.01$) at birth and more of their mothers with a history of asthma ($p = 0.007$). Logistic regression analysis including these variables did not affect the differences in cytokine profiles IL-5 and IL-2 between wheeze and healthy as well as eczema and healthy groups. However, the association of IFN-γ with wheeze was no longer significant ($p = 0.05$) but the association of IL-13 with wheeze became statistically significant ($p = 0.047$) after adjustment (Table 2).

Logistic regression was also used to determine separately the main predictors of infant wheeze (i.e. wheeze outcome as the dependent variable). All clinical variables and cytokine profiles that were statistically different between groups in the univariate analysis were entered into the logistic regression models (Figure 3). In this cohort, logistic regression analysis determined that higher levels of IL-5 (OR, 35; 95% CI, 5.0 – 246.8), a child being the second born and younger siblings (OR, 10.3; 95% CI, 2.3 – 46.4), mothers with a history of asthma (OR, 4.5; 95% CI, 1.1 – 17.5), and an increased birth weight (OR, 8.46; 95% CI, 2.1 – 33.9) were factors associated increased risk for wheeze ($p < 0.05$) (Figure 3).

Discussion

This study evaluated CBMC mitogen PHA stimulated cytokines responses in an at risk (first degree relative with allergic disease) birth cohort with distinct outcomes of wheeze or eczema at 24 months. The majority of those with outcomes of eczema 20/29 (69%) and 31/34 (91%) wheeze at 24 months of age were non-atopic (not sensitized to common environmental and food allergens). Our study is unique in that we were able to compare the outcomes of early onset wheeze and eczema as individual outcomes in the same birth cohort. We have chosen to study Th1 (IFN-γ and IL-2) and Th2 (IL-13 and IL-5) cytokines that are most commonly reported to be associated to viral wheeze.[8,10,11,12] From this study we observed that the wheeze subjects had a hyper-responsive T-cell cytokine profile seen from increased PHA stimulated CBMCs IL-2, IFN-γ and IL-5 responses, which was not seen in the eczema subjects (Figure 2). These data suggests that pre-existing hyper-responsive T-cell cytokine responses from birth may influence the development of early onset wheeze but not the onset of eczema.

We also observed that the development of wheeze was most strongly associated with increased Th2 cell cytokine IL-5 responses (Figure 2 and Figure 3). Zhang et al. studying a high risk cohort has reported that the high production of IL-5 was a predictor of respiratory infections that cause wheeze

Table 2. Association between cytokine responses from cord blood mononuclear cells with wheeze and eczema after logistic regression adjustment.

Cytokines		Phytohaemagglutinin (T cell mitogen) Median (IQR) pg/mL		Adjusted *p* value	OR (95% CI)	Phytohaemagglutinin(T cell mitogen) Median(IQR) pg/mL		Adjusted *p* value	OR (95% CI)
		Eczema (n=29)	Healthy (n=65)			Wheeze (n=34)	Healthy (n=65)		
Th1	IL-2	852 (271-1,588)	1,228 (290-2,400)	0.831	1.07 (0.58 - 1.98)	3,381 (1,780-4,914)	1,228 (290-2,400)	**0.021**	2.44 (1.14-5.22)
	IFN-γ	962 (403-2,022)	751 (326-1,859)	0.495	1.31 (0.60- 2.88)	2,708 (962-4,278)	751 (326-1,859)	0.050	2.50 (0.97-6.20)
Th2	IL-5	10 (10-30)	13 (10-26)	0.826	1.22 (0.20 -7.40)	29 (15-50)	13 (10-26)	**0.002**	16.8 (2.73-103.9)
	IL-13	3,395 (1,915-4,830)	3,919 (2,698-4,837)	0.078	0.25 (0.05-1.17)	4,461 (3,448-7,631)	3,919 (2,698-4,837)	**0.047**	1.02 (1.00-1.04)

Statistically significant differences ($p < 0.05$) are highlighted in bold.

in children.[11] The release of IL-5 has been shown to be responsible for airway inflammation, bronchial hyper-responsiveness and eosinophil recruitment in subjects with viral-induced wheezing.[11,17]

Furthermore, the increased production of cytokine IL-2 in cord blood cells of wheezing infants indicates that this cytokine may also be important in the pathogenesis of wheeze in early life. Although, the association of cytokine IL-2 in cord blood has not been previously reported in subjects with wheeze, IL-2 has been shown in rats to increase airway response when challenged with allergen ovalbumin.[18] Additionally, the peripheral blood mononuclear cells of patients with recurrent wheeze after respiratory syncytial virus (RSV) infections have been found to have increased allergen specific IL-2 response compared to the asymptomatic group.[19]

Early-life IFN-γ production has been reported to be inversely related to early onset wheezing. Guerra et al. has showed that impaired production of IFN-γ at 3 months of life is related to recurrent wheezing in the first year of life.[20] Similarly, Stern et al. reported that low IFN-γ production in 9-month olds was associated with an increased risk for wheeze at 6 years of age and this observation was independent of allergen sensitization.[21] Additionally, PHA stimulated cord blood in a study by Gern et al. showed that children in the first year of life with ≥ 2 episodes of wheeze had a lower production of IFN-γ.[22] The reduced CBMCs IFN-γ responses with PHA have also been observed in earlier studies of atopy.[12,23] In contrast, however, there were two other studies that showed no overall association between cord blood IFN-γ responses and wheezing at 1-[20] and 2-year of life.[24] Our study appears to be the first to report the association between increased IFN-γ levels and the outcome of wheeze by 2 years of age. Our observations are consistent with studies on the role of IFN-γ in the development of airway hyper-responsiveness in murine models.[25] Bronchial hyper-responsiveness in children has also been associated with increased IFN-γ, particularly from $CD8^+$ T cells.[26] Furthermore, the contrasting results in our study compared to what others have found could be due to that fact that almost half 16/34 (47%) of the wheeze subjects in this cohort wheezed only once, unlike the subjects studied by Gern et al.,[22] and only 3/34 (8.8%) of these subjects are atopic wheezers which makes our group of subjects different from those studied by Herbeth et al.[12] and Prescott et al.[23]

Our data points to hyper-responsive production of cytokines from CBMCs in the wheeze group that was not seen in the eczema group. Instead, the eczema group has exhibited a hypo-responsive cytokine profile compared to the wheezing subjects. The reduced cytokine production from CBMCs in eczema has been highlighted in a few studies. In our previous study, a hypo-responsive lipopolysaccharide stimulated IL-8 cytokine profile was observed in the eczema subjects compared to healthy subjects.[27] In addition, a study by Woods et al. found an association between reduced CBMCs IL-8 and IL-13 cytokine responses to a variety of innate, adaptive and mitogenic stimuli and have reported hypo-responsiveness of CBMCs stimulated with either lipopolysaccharide or peptidoglycan with lower IL-12, IFN-γ, IL-10 and TNF-α associated with the development of atopic dermatitis, compared to the healthy controls.[28] A few other studies have also reported reduced IFN-γ,[10,15] enhanced IL-4[12]

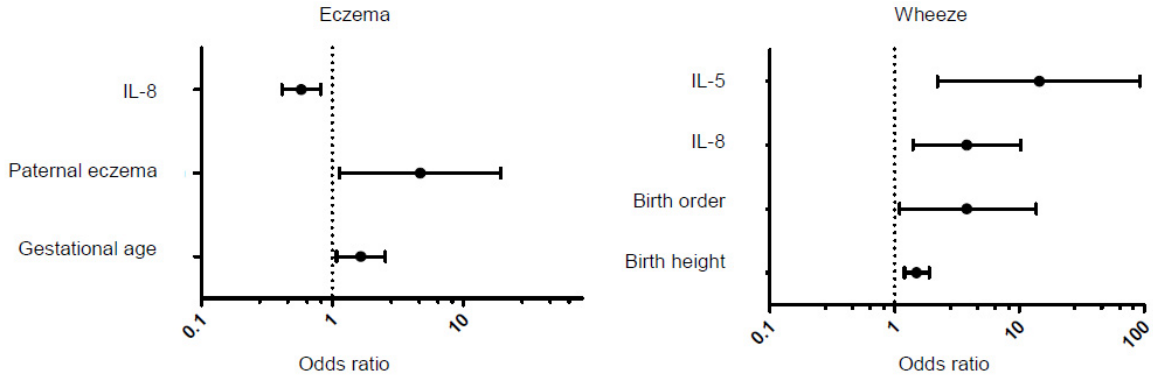

Figure 3. Relationship between the outcome of wheeze with cord blood cytokine profiles and clinical characteristics. Closed circles represent statistical significance when all clinical variables and cytokine profiles were included in the logistic regression model. Clinical factors analyzed were: mode of delivery, maternal asthma, birth order, birth weight and birth length.

and IL-13 to be associated with atopic dermatitis.[29] The discrepancies in our eczema cytokine profiles compared to the ones previously published could be due to the inclusion of all eczema subjects into their analyses, regardless of the sensitization status and their selection criteria for inclusion in the analyses, which might affect the cytokine production. For example, Wood et al. only included subjects with an eczema diagnosed with an EASI score greater than or equal to 1.0 at either the 3 or 12 month examination into their analyses. Furthermore, Prescott et al. has reported decreased levels of PHA stimulated IFN-γ and increased responses from lipopolysaccharide stimulated IL-6 and TNF-α in subjects who developed allergic manifestations (including food allergy or allergen sensitization) with the main outcome being eczema.[23] However, in the latter report, infants with concomitant wheeze were also included in the analysis and the data included both wheeze and eczema outcomes.[23] This may at least in part explain the hyper-responsive rather than hypo-responsive innate cytokine responses seen in their study.

Aside from the role of the immune system, our study also suggests that the second born children and younger siblings are more prone to the development of wheeze (Figure 3), and it has been reported by Perzanowski et al. that increasing birth order was associated with increasing prevalence of respiratory symptoms including wheeze.[30] Additionally, the birth weight of infants was found to be an independent risk factor associated with wheeze. This may be related to the observations that later born children tend to be heavier and have increased birth lengths.[31] A study in New Zealand found that the development of asthma was associated with increased birth length.[32] In contrast, a study in Stockholm found birth length to be inversely associated with any wheeze at age 4 years and late onset wheeze.[33] The association of birth weight and length with the outcome of wheeze is therefore not conclusive.

We recognize the limitations to our study. Firstly, the relatively small sample size of this study does not allow these findings to be generalized to the broader community. Secondly, wheezing symptoms were used as the outcome of our analysis because the diagnosis of asthma is difficult in early childhood; however 47% of the wheeze subjects in our study wheezed only once by 2 years of age and these children might be the ones with transient wheezing episodes. Further follow-up of this cohort is needed to determine if the current cytokine profile observed will be associated to the development of asthma or frequent wheezing later on life.

The culture conditions of our study might vary from other cord blood studies due to the fact that our cord blood cells were cultured in AIM-V serum free media, unlike other studies that have used RPMI-1640 supplemented with fetal calf sera[23] or pre-primed CBMCs with IFN-γ.[34] The different cell culture conditions used,[12,28] as well at the time point of the culture supernatant collection,[29] could affect the cytokine profile. A variety of cell culture conditions have been used in previous cord blood studies involving PHA as a stimulus and in our study we used a PHA concentration of 5 μg/mL with

a supernatant collection time point of 48 hours. A PHA concentration of 5 μg/mL was used in a study by Copenhaver et al.[35] and 1 μg/mL was used in studies by Rowe et al. and Zhang et al.[11,36] These studies all used the time point of 48 hours for supernatant collection.

In conclusion, our study suggests that there are inherent aberrant immunological responses involving cytokines from naive cord blood T cells that may influence the development of wheeze in early life. These responses were not observed for our subjects with eczema, even though eczema is a well documented risk factor for wheeze and subsequent asthma.[3] Taken together, early onset wheeze may be influenced at least in part by the presence of immune factors that are independent of eczema.

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Conflict of interests

The author(s) declare that they have no competing interests.

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