

ORIGINAL ARTICLE

Early onset wheeze associated with enhanced combined IL-1 β , IL-6, and IL-12/IL-23p40 in LPS-stimulated cord blood mononuclear cells

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Summary

Background Neonates with a family history of atopy are at higher risk for developing wheezing in early life.

Objective From a birth cohort of at risk infants (first-degree family with atopic disease), we evaluated the influence of distinct intrinsic immunologic risk factors on wheezing disorders in the first 2 years of life.

Methods Cord blood samples were collected from 195 eligible subjects of a birth cohort of 253 subjects. The subjects studied were those who developed wheezing ($n = 34$) or eczema ($n = 29$) in the first 2 years of life, and 65 healthy control infants. At the time of thawing the viability of the cells were median 70% (range 67.5%–72.5%). Cytokines from lipopolysaccharide (LPS)-stimulated mononuclear cells were analysed using fluorescent-activated cell sorting-array and their profiles were evaluated using factor analysis.

Results Infants with wheeze were significantly associated with enhanced combined LPS stimulated IL-1 β , IL-6, and IL-12/IL-23p40 compared with healthy controls ($P = 0.003$). This profile was also associated with the increased risk for wheeze at 2 years of age (OR = 2.45; 95% CI = 1.50–3.93, $P = 0.001$). LPS-stimulated cytokine IL-8 was also significantly higher in the wheeze group compared with healthy controls and eczema ($P = 0.003$). Intracellular staining showed that monocytes are main producers of IL-6 and IL-8 from cord blood mononuclear cells. Most of the subjects were non-atopic with 3/34 (9%) wheeze and 9/29 (31%) eczema subjects sensitized to the common dietary or inhalant allergens.

Conclusion and Clinical Relevance In infants at genetic risk of atopy, wheeze but not eczema in the first 2 years of life is associated with intrinsic hyperresponsive innate cytokine responses which might predispose infants to wheeze development. Distinct pre-symptomatic hyperresponsive innate immune responses risk factors were found to be associated with early onset wheeze disorders, but not eczema.

Keywords cord blood mononuclear cells, cytokines, early onset wheeze, infant eczema, innate immunity

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Introduction

Early childhood wheeze represents the most common cause of hospitalization of infants [1]. Wheezing before the age of 3 years and persistent childhood wheezing are more frequent in infants with family history of atopy [1–3]. The pathology of wheeze that influences the airway function involves epithelial sloughing, increased mucus production in airways, developmental and mechanical factors in the neuromuscular apparatus [4], plus aberrant immune responses that are likely to be involved [5–7]. The

intrinsic differences in the immune system not only predisposes infants to the development of wheeze but could also be involved in increasing its severity [6]. The innate immune response is of interest as the immaturity of the infant immune system is capable of altering the outcome of microbial, particularly viral, infections; leading to more severe forms of illness due to the aberrant immunological responses through secretion of cytokines and interaction of ligands [1].

Immunological responses are often associated with heterogeneous T-helper subsets such as Th1, Th2, and

Th17 contributing to the immunopathology of various inflammatory diseases [8]. Specific cytokine combinations in the microenvironment are necessary to induce the differentiation of naïve T cells in the umbilical cord blood into its respective T effector cells [9, 10]. Studies have shown that CD161⁺ CD4⁺ naïve T cells present in the human umbilical cord blood can give rise to Th17, Th1, and Th17/Th1 cells in response to IL-1 β and IL-23, and can also differentiate into Th1 cells, in response to IL-12, or to Th2 cells in response to IL-4. Whereas all the other CD161⁻ CD4⁺ naïve T cells can only differentiate into Th1 or Th2, but not Th17, cells [9].

In order to determine whether distinct profile of innate immune responses is an inherent feature in infants with wheezing disorders, this study set out to evaluate the immunophenotypes of cord blood mononuclear cell (CBMC) immune responses in a clinically well-defined birth cohort of at risk infants (first-degree relative with allergic disease) [11]. Subjects were grouped according to their clinic outcomes at 2 years of age: wheeze, eczema (serving as a disease-related control), and healthy controls. Cytokine, chemokine and soluble mediator responses of CBMC to toll-like-receptor 4 (TLR4) agonist lipopolysaccharide (LPS) were assessed to target innate antigen presenting cells. Taken together, the data from our study supports a new notion that an intrinsic hyper-responsive LPS-induced innate cytokine profile involving enhanced combined IL-1 β , IL-6, and IL-12/IL-23p40, is characteristic of early onset wheezers but not the eczema phenotype.

Methods

Subject recruitment criteria

Between May 2004 and June 2006, 253 families with a first-degree relative with allergic disease were recruited from the antenatal clinics at the National University Hospital to this study. Subjects from this cohort were originally participants of a probiotic intervention clinical trial (ClinicalTrials.gov Identifier: NCT00318695). Details of the inclusion/exclusion criteria and characteristics of the cohorts have been published previously [11]. Written informed consents were obtained from all families. The study was approved by the National University Hospital's ethics review committee (Ref Code: B/00/322) [11].

Follow-up examination

The primary clinical outcome measure was development of eczema and episodes of wheezing which were diagnosed by clinician's [11]. Wheezing episodes of each subject was recorded from hospital records for hospitalized infants and clinic records of specialists or family practitioners for those managed as outpatients. 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 for infants [12], and the severity assessed by The Scoring Atopic Dermatitis (SCORAD) index [13].

Serum was collected at 12 months of age and stored at -70°C before assayed for total IgE using the fluoroenzyme immunoassay method (UniCAPs Phadiatop, Pharmacia Diagnostics, Uppsala, Sweden) with a detection limit of 0.35 kU/L. Skin prick test was 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].

Preparation of cord blood mononuclear cells

Umbilical cord blood was collected into blood bags (Terumo, Somerset, NJ, USA) containing heparin and RPMI medium (Gibco Life Technology, Grand Island, NY, USA) and processed within 24 h. CBMCs were isolated by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) gradient centrifugation and cryopreserved in 14% dimethyl sulphoxide (Sigma, Munich, Germany) in foetal calf serum and stored in liquid nitrogen (-150°C) until analysis.

Stimulation of cord blood cells with lipopolysaccharide

After thawing, CBMCs were plated in 96-well round-bottom cell-culture plates in duplicates at 2×10^5 viable cells/well in 200 μl medium with AIM-V medium (Gibco Life Technology). At the time of thawing the viability of the cells were median 70% (range 67.5%–72.5%). Cells were cultured with or without of LPS at 1 $\mu\text{g}/\text{mL}$ (*Escherichia coli* LPS 0111:B4, Sigma) for 24 h at 37°C in 5% CO_2 , the culture supernatants were harvested and stored at -80°C . Cytokine profiles were analysed by fluorescence-activated cell sorter (FACS) – Array (Becton Dickinson, Franklin Lakes, NJ, USA). Optimal concentration of stimulants used and time-points of co-culture with CBMCs were evaluated based on initial experiments (data not shown).

Intracellular staining and flow cytometry

CBMCs from subjects with wheeze ($n = 3$) were cultured with 1 $\mu\text{g}/\text{mL}$ LPS for 24 h, and monensin (2 μM) (Sigma) was added for the last 16 h. Cells were then stained with surface markers anti-CD3⁺ PerCP, anti-CD14⁺ FITC, anti-CD19⁺ APC (Becton Dickinson) for 30 min. Intracellular cytokine staining was performed using an intracellular staining kit (Becton Dickinson), with specific monoclonal antibodies against the human cytokines IL-6 and IL-8 (Becton Dickinson). Fluorescence-labeled cells were analysed by the FACS Calibur (Becton Dickinson).

Cytokine protein detection

Detection of cytokine production was performed using the fluorescence-activated cell sorter (FACS) – Array (Becton Dickinson). Cytokines measured were IL-10, IL-1 β , IL-6, IL-8, IL-12p70, TNF- α , IFN- γ , IL-2, IL-5, IL-12/IL-23p40, and IL-13 using the BD bead array flexset (Becton Dickinson) with a detection limit of 10 pg/mL for all cytokines. The responses presented are those evoked by the LPS stimulus after subtraction by responses from unstimulated cultures.

Statistical analysis

Statistical analysis was performed using SPSS software version 16.0 for windows (SPSS Inc., Chicago, IL, USA). The Mann-Whitney *U* test was performed to assess the differences in cytokine responses between the three groups, healthy, wheezing, and eczema. The *P*-value was adjusted based on the Bonferroni correction to account for multiple comparisons. Differences in the demographic data were evaluated using the Mann-Whitney *U* and the χ^2 tests. Significant differences in groups at univariate analysis were included into the logistic regression to adjust for these confounders. Values of $P \leq 0.05$ were considered statistically significant. All figures were drawn using Sigma Plot (Systat Software Inc., Chicago, IL, USA).

Factor analysis was used to determine predictors of cytokines that were associated with wheeze and eczema. This analysis is a data reduction technique which enables a large set of variables (cytokines) to be grouped into intercorrelated sets of variables (cytokines) using statisti-

cal software SPSS. Factors with an eigenvalue greater than 1 were formed using only the healthy controls to describe cytokine profile of the healthy children. Factor-specific loading was calculated for each original variable which indicates the degree of correlation between the specific factor formed and the variable. To perform additional analysis by using factors as variables, factor scores were generated by the statistical software SPSS. Factor scores are a linear combination of all the variables weighted by the corresponding factor loadings. These factor scores were then compared between groups by Mann-Whitney test. Logistic regression was used to determine factor scores as possible predictors of wheeze and eczema while adjusting for confounding factors [15].

Results

Demographic characteristics

Of the 253 subjects at risk of allergy recruited a total of 195 cord blood samples were collected for the study. Of these, 178 samples had sufficient cells for evaluation but 50 were excluded from the study. Based on their clinical outcome, 128 subjects were divided into three groups (Fig. 1). Group 1: healthy control infants ($n = 65$) with no clinical manifestations of eczema, wheeze, or any atopic disorder, and were not allergen sensitized to dietary or inhalant allergens; Group 2: infants with wheeze ($n = 34$); and Group 3: infants who developed eczema ($n = 29$). There were only three (9%) subjects from the wheeze group and nine (31%) subjects from the eczema group who were sensitized to the common

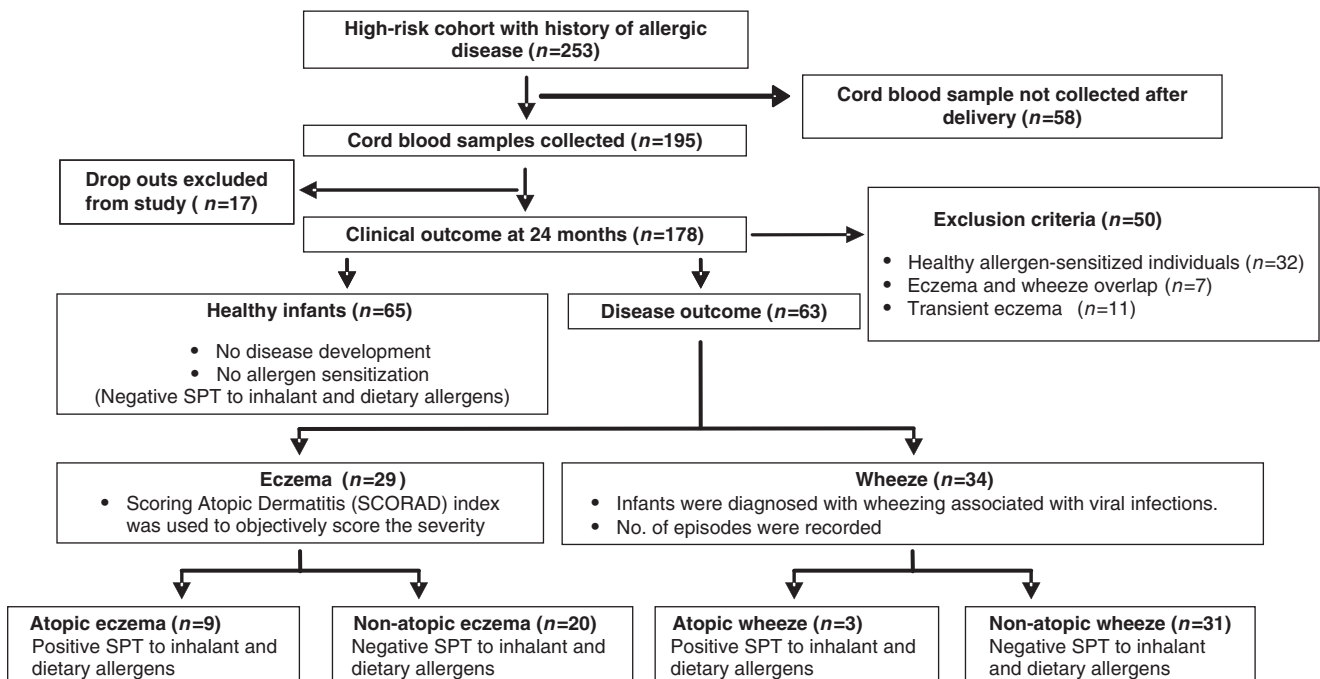


Fig. 1. Flow chart of study profile.

dietary or inhalant allergens (Table S1 in the supporting information). There were 50 subjects excluded from the study; and these were those with allergen sensitization without any clinical manifestation of atopy ($n = 32$), overlap eczema and wheeze ($n = 7$); transient eczema in early infancy ($n = 11$). In the Group 2 wheeze subjects, 24% infants developed wheeze below 6 months of age, 32% between age 13 and 24 months and the majority, 44%, between 7 and 12 months of age (Table S2 in the supporting information). There were 16 subjects with a single wheeze episode, while 14 subjects between two and three times, and only four with more than three times (Table 1). Notably, from the demographic characteristics a higher proportion of wheeze subjects had a family history of maternal asthma compared with the healthy controls (12/34 vs. 8/65, $P = 0.007$).

In the Group 3 eczema subjects, the majority (76%, $n = 22$) developed eczema by the age of 6 months (Table S2 in the supporting information). The mean SCORAD index of the infants with eczema at 24 months was 16.3. Additionally, a higher proportion of eczema subjects had a family history of paternal eczema compared with the healthy controls (24/29 vs. 8/65, $P = 0.027$) (Table 1). Group 3 was included in this study as a disease control group as eczema is a known risk factor to the development of wheeze in infants [16].

The total IgE geometric mean (95% confidence interval) for healthy controls, wheeze, and eczema groups was 15.8 (11.7–21.4) kU/L, 27.2 (16.9–43.9) kU/L, and was 24.5 (13.3–45.4) kU/L; respectively ($P > 0.05$).

Cytokine responses in lipopolysaccharide-stimulated cord blood mononuclear cells

The cytokine profiles are shown in Fig. 2 and expressed as medians and interquartile ranges. Both the wheeze 47.4 (17.0–85.1) ng/mL and eczema 23.1 (17.6–33.7) ng/mL groups produced higher levels of IL-6 compared with healthy controls 15.9 (7.90–25.5) ng/mL, ($P = 0.003$ and 0.036, respectively) with the wheeze group producing the highest levels (wheeze vs. eczema, $P = 0.024$). In addition, LPS stimulated IL-12/IL-23p40 production was also significantly higher in the subjects with wheeze compared with the healthy controls 0.10 (0.04–0.17) vs. 0.04 (0.01–0.08) ng/mL, $P = 0.003$. Notably, the readout for the related cytokine IL-12 (shared p40 subunit), IL-12p70, was undetectable in all groups. Excluding those with allergen sensitization in Groups 2 and 3 did not affect the results (data not shown). Likewise IL-8 responses in wheeze 182.4 (80.4–252.7) ng/mL subjects were also significantly higher compared with both healthy controls 94.5 (12.6–156.6) ng/mL and eczema subjects 12.1 (2.80–76.7) ng/mL ($P = 0.003$, respectively). In contrast, the eczema subjects had significantly attenuated IL-8 production compared with both the healthy controls and wheeze subjects ($P = 0.018$ and 0.003, respectively).

There were no significant differences between groups for the production of other cytokines (IL-1 β , TNF- α , and IL-10) when evaluated individually. IFN- γ , IL-13, IL-5, IL-2 were undetectable in the LPS-stimulated groups (Table S3 in the supporting information).

Logistic regression analysis of cytokine profiles

To adjust for potential confounding demographic and clinical factors, univariate analysis of the variables (Table 1) was evaluated. Based on factors that were statistically different between groups, the comparison of cytokine profiles were adjusted for the following variables: gestational age and paternal eczema for eczema and control groups; and mode of delivery, maternal asthma, birth order and birth weight and birth height for the wheeze and control groups. Logistic regression analysis including these variables did not affect the differences in cytokine profiles between groups (Table S3 in the supporting information). Similar variables were used for adjustment of factor scores in the logistic regression with eczema or wheeze as dependent variables.

Factor analysis of cytokine profiles

Factor analysis of LPS-stimulated cytokine responses showed that infants with wheeze have higher factor scores with combined IL-1 β , IL-6, and IL-12/IL-23p40 responses compared with healthy control subjects and eczema subjects ($P = 0.003$) (Table 2). Factor 1 indicates that IL-1 β , IL-6, and IL-12/IL-23p40 are correlated variables. These three cytokines in combination are also significantly enhanced in the wheeze group (Table 2), even though as individual cytokines, only IL-6 and IL-12/IL-23p40, but not IL-1 β , are significantly higher in the wheeze group. Logistic regression analysis showed that the combined cytokine responses (IL-1 β , IL-6, and IL-12/IL-23p40) could predict wheeze relative to the scores for the healthy control subjects (OR, 2.45; 95% 1.50–3.93, $P = 0.001$). When the three variables selected by the factor analysis were presented using a 3D plot, the wheeze group showed a positive correlation between these cytokines (Fig. 4). Whereas, combined responses from TNF- α , IL-10, and IL-8 had lower factor scores (lower production) in the eczema group compared with the healthy control group ($P = 0.003$) and wheeze group ($P = 0.003$) (Table 2).

Monocytes are main producers for IL-6 and IL-8 in lipopolysaccharide-stimulated cord blood mononuclear cells

Intracellular cytokine staining analysis identified monocytes (CD14⁺ cells) as the main cell type that produces IL-6 and IL-8 (~twofold higher post LPS stimulation) but not CD19⁺ B cells (Fig. 3) or CD3⁺ T cells (data not shown).

Table 1. Demographic characteristics of the children with eczema and wheeze and the healthy control subjects

	Healthy (<i>n</i> = 65)	Eczema (<i>n</i> = 29)	Wheeze (<i>n</i> = 34)	Healthy vs. eczema (<i>P</i> -value)	Healthy vs. wheeze (<i>P</i> -value)
Gestational age in weeks median (IQR)*	38.7(1.6)	39.6(1.7)	39.3(1.9)	0.021	0.088
Infant sex, no (%)					
Male	30(46)	18(62)	19(56)	0.154	0.358
Ethnicity (%)				0.946	0.873
Chinese	30(46)	14(48)	13(38)		
Malay	27(42)	12(41)	16(47)		
Indian	7(10)	3(10)	4(12)		
Others	1(2)	0(0)	1(3)		
Mode of delivery, no (%)					
Lower segment caesarean section*	20(31)	6(21)	4(12)	0.313	0.036
Family history first-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*				0.771	0.038
1	27(42)	14(45)	7(23)		
2	13(20)	6(20)	14(45)		
> 3	25(38)	9(35)	13(32)		
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
Weaning age in months mean (SD)	6(1)	6(0.0)	6(2.0)	0.055	0.378
Prenatal maternal smoking	4(6)	2(7)	2(6)	1.000	1.000
Passive smoke exposure in household	19(29)	9(31)	14(41)	0.86	0.231
Keep pets (%)	18(28)	5(17)	6(18)	0.276	0.268
Type of housing (%)				0.626	0.306
Public housing	56(86)	26(90)	26(76)		
Private housing (condominium)	5(8)	2(7)	8(24)		
Landed property	4(6)	1(3)	0(0)		
Feeding history – birth to month 6 (%)				0.187	0.761
Near total breastfeeding with at least 60 mL of formula for 6 months	1(2)	2(7)	0(0)		
Any breastfeeding	47(72)	19(66)	25(74)		
Total formula	17(26)	8(28)	9(26)		
Probiotic supplementation	30(46)	9(31)	14(41)	0.169	0.636
SCORAD index Mean±SD (range)†	0	16.3±8.1 (3.7–35.9)	0		
Frequency of wheeze					
1	0	0	16		
2–3	0	0	14		
> 3	0	0	4		

*Statistically significant differences in variables between groups.

†SCORAD was not recorded for 13 subjects in the eczema group.

Discussion

Functional alterations of the neonatal antigen presenting cells (APCs) such as monocytes/macrophages and dendritic cells (DCs) could lead to secondary effects such as

hyper-responsiveness of the adaptive T cell immunity and therefore susceptibility to disease [1, 8]. In our at-risk for atopy birth cohort [11], we prospectively assessed the relationship of the immunophenotypes in CBMCs and the subsequent development of wheeze and eczema in their

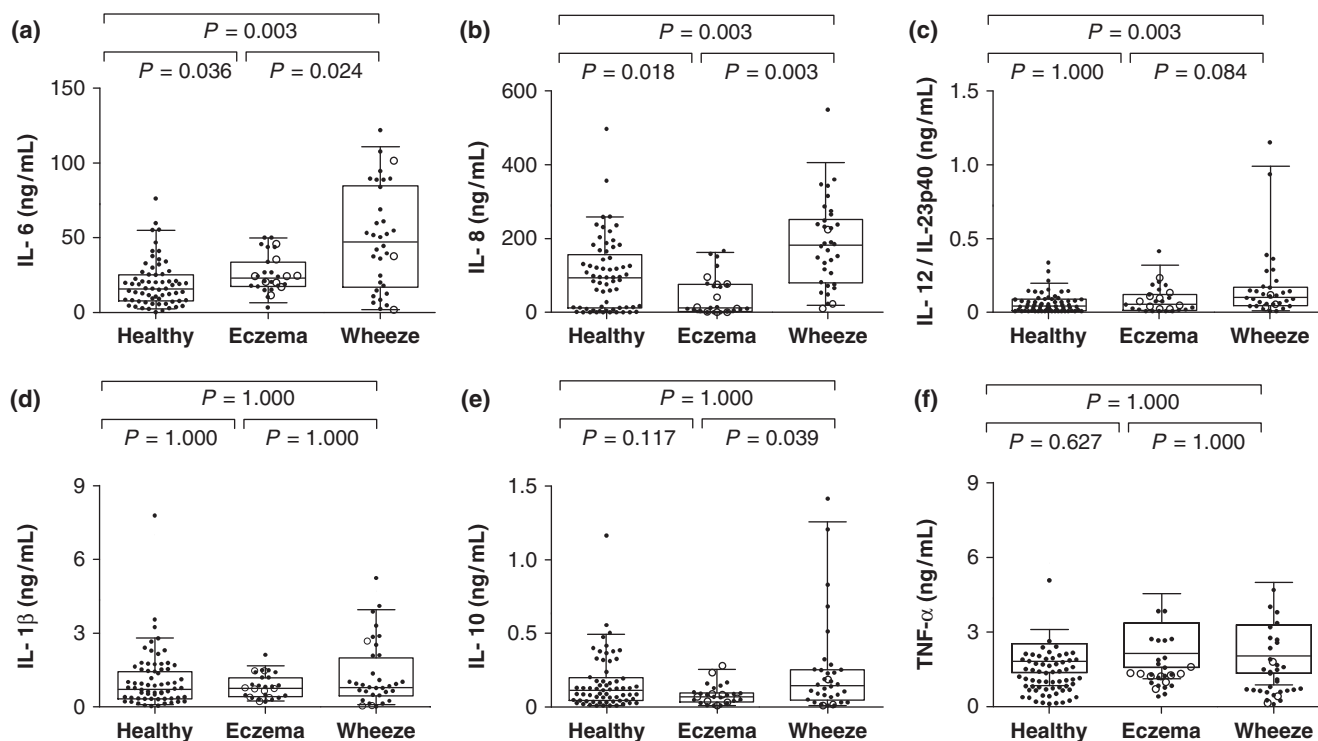


Fig. 2. (a–f) CBMC cytokine profile of healthy ($n = 65$), eczema ($n = 29$) and wheeze ($n = 34$) subjects in response to $1 \mu\text{g/mL}$ LPS. Closed circles ($\bullet$) represents non-allergen sensitized subjects, while the open circles ($\circ$) represents allergen sensitized subjects. The band in the middle of the boxplot represents the median value, and the ends of the whiskers represent the 5th and 95th percentile value of the data.

first 2 years of life. Using factor analysis, we made a novel observation that infants with early onset wheeze correlated with enhanced combined factor of LPS induced IL-1 β , IL-6, and IL12/IL-23p40. Additionally, this factor was highly associated with the increased risk for early onset wheeze (Table 2, Figs 2 and 4). The increased combined cytokine production capacity by LPS-stimulated APCs (macrophages/monocytes/DCs) is of particular interest as this combination of cytokines is an important determining factor for the differentiation of IL-17-producing Th17 cells in humans [17–19].

IL-23 is a member of IL-12 cytokine family. It is a heterodimeric cytokine produced by antigen presenting cells (monocytes and DCs) comprising of p19 subunit specific for IL-23 and a p40 subunit shared with IL-12 [20]. The secretion of IL-12(p70) in neonatal dendritic cells is impaired due to a specific defect in the IL-12(p35) subunit transcription, while the IL-12(p40) subunit transcription is preserved [21]. Hence, neonatal monocytes are biased in the production of IL-23 [21, 22]. We therefore reason that it is likely the IL-12/IL-23p40 measured in this study could be from IL-23. The combination of IL-23 plus IL-1 β is crucial for the differentiation of CD161 $^{+}$ precursor T cells from cord blood and newborn thymus into IL-17A producing Th17 cells, and for polarizing neonatal CD4 $^{+}$ and CD8 $^{+}$ T cells into IL-17 producing cells [18, 19, 22, 23]. In addition, pro-inflammatory cytokines IL-6 and

Table 2. Summary of factor analysis, including factor loadings from a model of cytokine responses in the LPS stimulated CBMCs of healthy control subjects

Cytokine responses in LPS-stimulated CBMC	
Factor 1	Factor 2
IL-1 β (0.776)	TNF- α (0.701)
IL-6 (0.644)	IL-10 (0.642)
IL-12/IL-23p40 (0.691)	IL-8 (0.769)
Total variance explained (%)	
33.50	26.50

CBMC, cord blood mononuclear cell; LPS, lipopolysaccharide

IL-1 β , through (STAT3), results in the up-regulation of the Th17-specific transcription factor, retinoic acid-related orphan receptor C (RORC2), which ultimately drives Th17 differentiation [17]. Furthermore, it has been shown that cytokines IL-6 and IL-1 β but not transforming growth factor are crucial for the differentiation of human Th17 cells. In addition, LPS-activated monocytes, but not the monocyte-derived dendritic cells, efficiently primed the differentiation of human Th17 via production of IL-6 and IL-1 β [17]. Moreover, a recent animal study using a viral PAMP double-stranded RNA also suggested that IL-6 plays a crucial role in the development of the Th17 cell response in the airways associated with viral respiratory

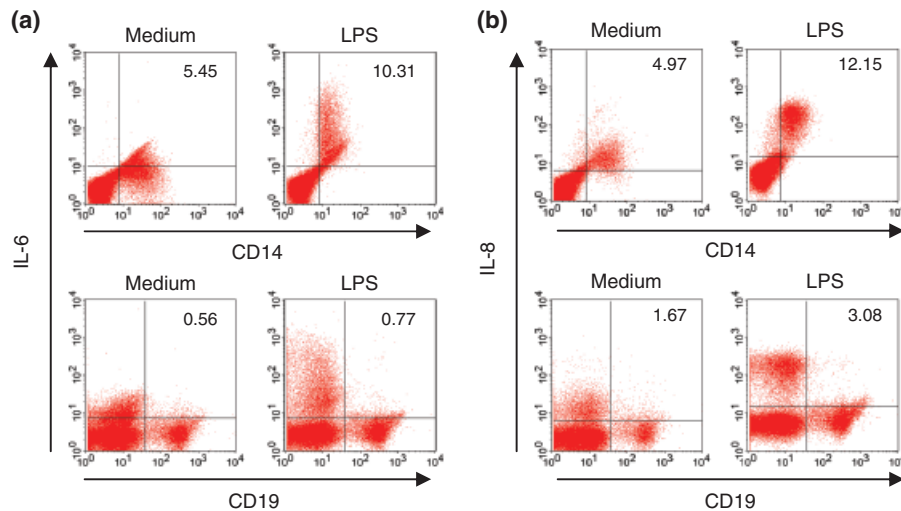


Fig. 3. Expression of IL-6 and IL-8 cytokine from lipopolysaccharide (LPS)-stimulated cord blood mononuclear cells (CBMCs) from one representative subject. Intracellular staining was performed to analyse the production of (a) IL-6 and (b) IL-8 cytokines by B-lymphocytes (CD19⁺) and monocytes (CD14⁺). Mononuclear cells were first gated according to the forward and sideward scatter. The up-regulation of IL-6 and IL-8 cytokines are depicted as percentage of CD14⁺IL-6⁺ cells and CD14⁺IL-8⁺ cells.

infection [24]. The importance of the innate immune regulation in immunopathology following viral infection has been highlighted in a study involving respiratory syncytial virus (RSV) infected (STAT-1) – knock-out mice. The attenuated type I interferon (IFN) (α/β) production was associated with increased mucus production and airway hyperresponsiveness along with increased pro-inflammatory cytokines IL-17 and IL-23 [20]. This study suggests that the IL-23/IL-17 cytokine axis plays a role in the pathogenesis of viral induced airway inflammation. Together, this line of evidence raises the possibility that the enhanced combination of responses of cytokines IL-12/23p40, IL-1 β and IL-6 in LPS-stimulated CBMC of infants with early life wheezing, may be indicative of an inherent feature of the innate system of these infants.

We also observed an increased IL-8 production by their LPS-stimulated CBMCs in the wheeze group (Fig. 2). These are consistent with previous studies showing higher CBMC production of IL-6 and IL-8 in response to stimulation with RSV [25, 26] or LPS [6]. In the physiological condition, IL-8 is a primary neutrophil chemokine attractant molecule secreted by epithelial cells upon viral infection [27], and is thought to play a role in viral-induced wheezing. For example, a study of the promoter region of IL-8 identified a common single-nucleotide polymorphism (SNP) at position –251 to be associated with RSV induced wheezing [28] and it has also been identified that whole blood from individuals carrying the (–) 251A allele produce greater IL-8 response when stimulated with LPS *in vitro* [29]. Most of our subjects who developed wheeze were non-atopic (91%) and their CBMC showed increased production of IL-8 upon stimulation with LPS.

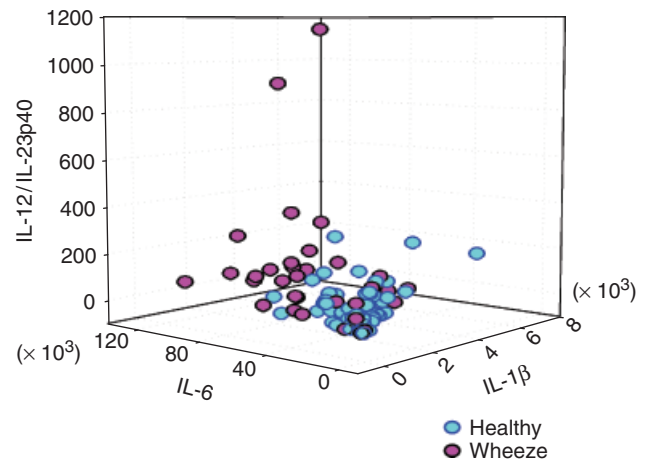


Fig. 4. 3D scatter plot of the healthy control and wheeze group in respect to their correlation to the variables from the factor analysis. Factor analysis of LPS-stimulated cord blood mononuclear cell (CBMC) showed that infant wheeze has higher combined responses from cytokines IL-6, IL-12/23p40, and IL-1 β compared with healthy controls. Figure was drawn with Sigma Plot (Systat Software Inc.).

IL-10 cytokine has been implicated to play an immunoregulatory role by inhibiting the progression or expression of inflammatory diseases [7, 30, 31]. However, in our study, IL-10 induced by LPS stimulation was not significantly different between wheeze and healthy groups (Fig. 2).

Additionally our data have identified monocytes from the CBMC to be the main cell type that produces IL-6 and IL-8 upon LPS stimulation (Fig. 3). It has been shown that LPS-activated monocytes produced significant levels of IL-6 and IL-1 β [17]. Furthermore, purified human CD14⁺

monocytes produced high levels of IL-23 in response to LPS [32]. In recent years, there have been a number of studies reporting phenotypically and functionally distinct subsets of human monocytes that play a key role in polarizing the innate and the adaptive immune system and act by regulating the adaptive responses in a type I or and type II direction [33, 34]. On the basis of this emerging evidence, we propose a notion that monocytes could be the major cell type involved in the intrinsic immune dysregulation in predisposing neonates to the development of early childhood disease. In the context of the current study, we proposed that the impaired functionality of monocytes at birth could be associated with the susceptibility to wheeze in early life.

The limitation in our current study is that the IL-12/IL-23p40 measured could be from cytokines IL-12/IL-23p40 homodimer, IL-12, or IL-23. Of these, IL-12 is unlikely, as we also measured IL-12p70 in supernatants of LPS-stimulated CBMC and found them to be below the detection limit (data not shown). The possibility of detecting IL-12/IL-23p40 homodimer cannot be ruled out.

In conclusion, our study suggests that an inherent immunological predisposition influences the development of wheeze in early life. While other studies have only focused on high- and low-risk neonates without follow-up on subsequent outcome of atopy/allergic diseases or examined the atopic phenotypes in isolation [35–38], in this study we have clearly evaluated the 2-year clinical

outcome of our cohort, compared the phenotypes of early life wheeze and eczema independently. In this study, most of the subjects were non-atopic as they are not sensitized to inhalant or dietary allergens by 2 years of age. Further clinical follow-up would determine if these children will develop atopy in the future. Technique-wise, we stimulated CBMCs using serum-free culture medium without pre-priming with IFN- γ to prevent the induction of other mediators that may confound the authentic cytokine profiles. These findings, therefore, provide the impetus to further define the functional aberrations of the innate immunity and the possible role of the IL-23/IL-17 axis associated with early onset wheezing.

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Conflict of interest: The authors declare that there are no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Allergen sensitization chart of infants at 24 months of age.

Table S2. Age of disease onset for the eczema ($n = 29$) and wheeze ($n = 34$) subjects.

Table S3. Cytokine responses to lipopolysaccharide (LPS) in infants with eczema and wheeze. P values have been adjusted for risk factors gestational age, paternal eczema

using logistic regression analysis with eczema as the outcome and for risk factors birth height, birth weight, birth order, mode of delivery and maternal asthma with wheeze as the outcome.

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Table S1: Allergen sensitization chart of infants at 24 months of age .

Allergen sensitization (%)	Healthy n=65 (%)	Eczema n=29 (%)	Wheeze n=34 (%)
Positive skin prick test	0(0)	9(31)	3(9)
Dietary (Any)	0(0)	1(3)	1(3)
Cow's milk	0	0	0
Egg white	0	1	1
Egg yolk	0	0	0
Soy	0	0	0
Inhalant allergens (Any)	0(0)	8(28)	3(9)
Dermatophagoides pteronyssinus	0	8	3
Blomia tropicalis	0	1	2
Multiple sensitized	0(0)	1(3)	1(3)
Monosensitized	0(0)	8(28)	2(6)

Table S2: Age of disease onset for the eczema (n=29) and wheeze (n=34) subjects

Age of onset (Months)	Eczema n=29 (%)	Wheeze n=34 (%)
< 6	22 (76%)	8 (24%)
7 up to 12	5 (17%)	15 (44%)
13 up to 24	2 (7%)	11 (32%)

Table S3: Cytokine responses to lipopolysaccharide (LPS) in infants with eczema and wheeze. P values have been adjusted for risk factors gestational age, paternal eczema using logistic regression analysis with eczema as the outcome and for risk factors birth height, birth weight, birth order, mode of delivery and maternal asthma with wheeze as the outcome .

Cytokines		Lipopolysaccharide (Innate stimulus)				Lipopolysaccharide (Innate stimulus)							
		Eczema Median (IQR) (pg/mL)		Adjusted P value		OR (Odds Ratio)		Wheeze Median (IQR) (pg/mL)		Adjusted P value		OR (Odds Ratio)	
		Eczema (n=29)		Healthy (n=65)				Wheeze (n=34)		Healthy (n=65)			
Pro-inflammatory	IL-6	23,136	(17,634-33,765)	15,930	(7,898- 25,476)	*0.024	6.442 (1.285-32.298)	47,404	(17,090-85,142)	15,930	(7,898- 25,476)	**0.003	8.554 (2.108-34.716)
	TNF-α	1,290	(695-1,921)	1,146	(691- 1,870)	0.133	2.809 (0.731-10.791)	1,219	(631-2,404)	1,146	(691- 1,870)	0.631	0.746 (0.225 - 2.470)
	IL-1b	829	(458-1,307)	783	(360- 1,584)	*0.455	1.617 (0.459 -5.696)	871	(484-2,209)	783	(360- 1,584)	0.4	1.563 (0.552 - 4.427)
	IL-8	12,050	(2,766-76,709)	94,547	(12,667-156,643)	**0.007	1.951 (1.205-3.160)	182,395	(80,432-252,740)	94,547	(12,667-156,643)	*0.037	3.505 (1.082-11.356)
	IL-12/IL-23p40	53	(13-119)	40	(10-87)	0.212	2.043 (0.666 - 6.271)	101	(43-169)	40	(10-87)	**0.008	5.481 (1.559-19.273)
Regulatory	IL-10	67	(34-95)	113	(42-197)	0.131	0.441(0.152-1.276)	145	(45-252)	113	(42-197)	0.666	1.251 (0.453-3.451)
Th1	IL-2	ND		ND				ND		ND			
	IFN-γ	ND		ND				ND		ND			
	IL-12p70	ND		ND				ND		ND			
Th2	IL-5	ND		ND				ND		ND			
	IL-13	ND		ND				ND		ND			

* Significant differences were found between groups with P value < 0.05

** Significant differences were found between groups with P value < 0.01

ND Not detectable