Original article

Cytokine responses in cord blood predict the severity of later respiratory syncytial virus infection

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Background: It has been claimed that an early respiratory syncytial virus (RSV) infection can induce asthma and recurrent wheezing.

Objective: We addressed the question of whether infants contracting an early RSV infection differ from healthy children in their cytokine production at birth.

Methods: In a prospective cohort study cord blood samples were collected from 1084 newborns during autumn 2001. Of 47 of these newborns with subsequent virologically confirmed RSV infection before 6 months of age, 24 had enough cells for stimulation in cord blood samples (14 of those were hospitalized). Twenty-eight children had other respiratory virus infections (16 with enough cells), and samples from 48 healthy children of the 1084 total served as control specimens. Stimulated cytokine production of mononuclear cells was measured. The responses in the groups were evaluated by means of factor analysis.

Results: The infants hospitalized for RSV infection had higher LPS-stimulated combined IL-6 and IL-8 responses than the infants treated as outpatients ($P = .005$) or the healthy control subjects ($P = .02$). The hospitalized patients with RSV showed lower IL-1β, IL-2, IL-4, IL-5, and IL-10 responses than those treated as outpatients ($P = .02$). High IL-6 and IL-8 responsiveness predicted a severe RSV infection (odds ratio, 2.20; 95% CI, 1.17-4.14; $P = .01$). The unstimulated cytokine responses at birth did not differ between the patients and healthy control subjects.

Conclusion: The results suggest that natural differences in innate immunity predispose children to severe RSV infection rather than the infection modifying immune responses in childhood. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Cord blood, cytokine, respiratory syncytial virus*

Abbreviations used
CBMC: Cord blood mononuclear cell
RSV: Respiratory syncytial virus

It has been claimed that children with a respiratory syncytial virus (RSV) infection during infancy have increased susceptibility to subsequent recurrent wheezing and asthma, but it is not known whether this tendency precedes the infection or is caused by it.[1-4] Individual differences in the immune system, such as in the capacity to produce cytokines, could affect the type of RSV-induced immune response and promote susceptibility to the infection and increase its severity. Alternatively, or even additionally, an RSV infection in early life before the immune system has fully developed could contribute to later susceptibility to asthma.

Analysis of cord blood leukocytes could provide an efficient means of gaining information on the immunologic capacity of an individual before any infection. Several studies have concentrated on the cytokine production of stimulated cord blood cells, but few of them have linked the findings to subsequent RSV infection among the same children.[5,6] We have previously observed that children with an RSV infection in early life differed from healthy control subjects in their later immune responses. In addition, RSV epidemics have not been associated with the subsequent use of asthma medication.[7,8] In a recent review no evidence was found for a causal association of RSV infection with later asthma.[4]

We report here on a prospective cohort study in which cord blood samples were taken from infants at birth and the same individuals were followed up clinically during infancy. Our aim was to find out whether infants hospitalized for an RSV infection during infancy differ from those with a milder RSV infection, those with other respiratory viral infections, or healthy control subjects with respect to the cytokine responses in their cord blood mononuclear cells (CBMCs). Our hypothesis was that differences in cytokine production in cord blood cells could affect the susceptibility to RSV infection and its severity.

Some of the results of these studies have been previously reported in the form of an abstract.[9]

METHODS

The study was carried out in the Department of Paediatrics at Oulu University Hospital, which is the only children's hospital in the area. In Finland RSV epidemics follow a 2–year cycle, beginning with a minor peak in the spring of an uneven year followed by a major peak during the next winter.[10] From August to November 2001, before the RSV epidemic season, a cord blood sample was collected from each newborn with at least 37 gestational weeks (n = 1084) whose parents provided informed consent (Fig 1). The

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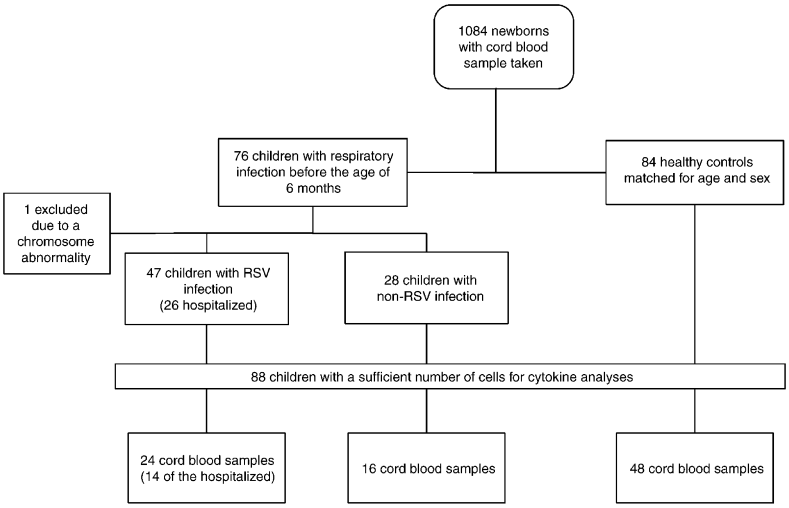

FIG 1. Flow sheet of the study profile. Cord blood data were not available for all the infants because of a lack of samples or inadequate amounts of mononuclear cells in some samples.

parents were asked to contact the authors immediately if their child presented with symptoms of respiratory tract infection before the age of 6 months and if they were interested in participating in the study. In the case of infection, the child was examined within 24 hours and admitted to the hospital, if necessary. The cause of the viral infection was determined by means of antigen detection from a nasopharyngeal secretion sample using an in-house viral antigen–specific enzyme immunoassay method and PCR (for detection of rhinovirus).[11,12] Children with a clinical respiratory tract infection and a positive result for RSV formed the initial study group (n = 48, 27 hospitalized [1 later excluded because of a chromosomal abnormality] and 21 treated as outpatients; mean age, 3.0 months; range, 3 weeks to 5.5 months). They were included in the study either according to parental contact or after being admitted to the hospital without prior contact with the authors. Of these 47 children, 24 had enough cells for stimulation in their cord blood samples, including 14 of those hospitalized for RSV infection. Those with a negative result for RSV (n = 28, 3 hospitalized; mean age, 3.1 months; range, 3 weeks to 5.7 months) formed one control group (16 with enough cells for stimulation). A group of healthy control subjects with no respiratory tract infections before the age of 6 months (as determined based on parental report, n = 84) and matched with the above for date of birth and sex was selected from those whose cord blood specimens were available (48 with enough cells for stimulation). None of the patients or control subjects required respiratory assistance during the neonatal period or had any concomitant disease, such as congenital heart disease or severe neurologic disease.

A family history of asthma and atopy was defined in terms of physician–diagnosed asthma, allergic rhinitis, atopic eczema, or food allergy in first-degree relatives, as reported by the parents.

Processing of the cord blood samples and cytokine analyses

Cord blood samples were centrifuged within 24 hours, and the CBMCs were separated and stored at −70°C until analysis. After thawing, CBMCs were plated in 24-well cell-culture plates at 2×10^6 viable cells/mL in RPMI 1640 medium supplemented with 0.6 mg/mL penicillin, 60 mg/mL streptomycin, 2 mmol/L L-glutamine, 20 mmol/L HEPES, and 5% FCS (Integro BV, Dieren, The Netherlands) and stimulated with LPS from *Escherichia coli* (100 ng/mL; HB101; Sigma, St Louis, Mo). After 24 hours' stimulation of mononuclear cells at 37°C in 5% CO_2, the culture supernatants were harvested and stored at −20°C for later cytokine determination.

Cytokine levels in the cell-culture supernatants were determined by means of flow cytometry with the human T_H1/T_H2 10plex Kit II (Bender MedSystems, Vienna, Austria), and the results are shown in picograms per milliliter (means ± 1 SD). The detection limits for cytokines were as follows: TNF-α, 7.9 pg/mL; IFN-γ, 7.0 pg/mL; IL-1β, 4.5 pg/mL; IL-2, 8.9 pg/mL; IL-4, 6.4 pg/mL; IL-5, 5.3 pg/mL; IL-6, 4.7 pg/mL; IL-8, 6.4 pg/mL; IL-10, 6.9 pg/mL; and IL-12p70, 9.7 pg/mL. The responses presented are those evoked by the medium alone and those produced after LPS stimulation after subtraction of the cytokine levels observed in unstimulated cultures.

The data were analyzed with SPSS for Windows, version 14.0 (SPSS, Inc, Chicago, Ill). The test for differences in proportions, the Student *t* test, and the Mann-Whitney *U* test were used to evaluate differences in the demographic data, and the Mann-Whitney *U* test was used to assess differences in the raw cytokine responses. The production of cytokines is expressed in picograms per milliliter ± SDs.

Because cytokines act in complex networks, we used factor analysis to combine cytokine responses that were closely intercorrelated to simulate a cascade type of cytokine activity. In factor analysis the statistical program used forms factors by combining those original variables that best correlate with the specific factors.[13] Factor-specific loading for each original variable indicates the degree of correlation between the specific factor formed and the variable, in this case the raw data from each assay. Loadings for each cytokine assay were estimated by using only the healthy control subjects to describe the cytokine profiles of healthy children. Standardized factor-score variables for each individual were derived from the loadings and interpreted as a summary of those original variables, with high factor loadings associated with the factors from which the scores were derived. The scores were calculated as weighted means of the standardized variables, standardization meaning that the resulting scores of the group of healthy children had a mean value of 0 and an SD of 1. Factors with an eigenvalue of greater than 1 were used for further comparison of the factor scores between the groups by means of the Student *t* test. The SPSS program is able to calculate the factor-score variables for both those used to estimate the original loadings (the cytokine responses of the healthy children) and those not involved in that process, making it possible to compare the groups. Logistic regression analysis was used to analyze the factor scores as predictors of susceptibility to RSV infection and of its severity while adjusting for confounding factors.

The protocol was found acceptable by the Ethical Committee of the Northern Ostrobothnia Hospital District. All the parents provided written informed consent.

RESULTS

Demographic characteristics

Demographic characteristics were analyzed in the groups, including all the children initially enrolled in the study. The

TABLE I. Demographic characteristics of the children with RSV and other respiratory virus infections and the healthy control subjects

	RSV (n = 47)		Control subjects with other viral infections (n = 28)*		Healthy control subjects (n = 84)		Difference (95% CI)
	Mean/no.	Range/%	Mean/no.	Range/%	Mean/no.	Range/%	RSV vs healthy†
Gestational age (wk)	39.8	37.4-42.3	39.6	37.6-41.7	40.1	37.1-42.3	−0.3 (−0.7 to 0.1)
Mode of delivery							
Vaginal	41	87	26	93	76	91	−0.03 (−0.17 to 0.08)
Cesarean section	6	13	2	7	8	10	
Birth weight (g)	3672	2850-4650	3739	3000-4790	3539	2640-5410	133 (−46 to 312)
Birth order	2.6	1-10	3.6	1-11	2.0	1-9	0.6 (−0.0 to 1.2)‡
Breast-feeding at the time of the infection§	36	77	26	93	53	82	−0.05 (−0.21 to 0.10)
Duration of breast-feeding (mo)‖	7.4	0-24	8.4	0.3-23	8.1	0-36	−0.7 (−2.9 to 1.5)
Mother smoking during pregnancy	10	21	4	14	9	11	0.11 (−0.02 to 0.25)
Smoking in the household at the time of the infection¶	20	43	6	21	16	34	0.09 (−0.11 to 0.28)
Family history of asthma or atopy¶	35	75	23	82	35	75	0 (−0.18 to 0.18)
Parental asthma¶	15	32	6	24	11	23	0.09 (−0.10 to 0.26)

Values are presented as means with range for continuous variables and numbers with percentages for categorical variables.
*No significant differences between the patients with RSV and the control subjects with some other viral infection.
†Student *t* test or test for a difference in proportions, where appropriate.
‡$P = .008$, Mann-Whitney *U* test.
§For healthy control subjects (n = 65 because of missing information) in the age corresponding to the time of the infection among subjects.
‖For duration of breast-feeding (n = 45 among the patients and n = 62 among the healthy control subjects because of missing information).
¶For healthy control subjects in the age corresponding to the time of the infection among patients (n = 47 because of missing information among those with permission only to use the cord blood sample, with no clinical follow-up, except for information about respiratory tract infections).

children with RSV infection and the control subjects with some other viral infection had a significantly higher birth order than the healthy control subjects (Table I). A smaller proportion of the infants hospitalized for RSV infection was still being breast-fed at the time of enrollment than of those treated as outpatients (16/26 vs 19/21, $P = .02$), the control subjects with some other viral infection (16/26 vs 26/28, $P = .005$), or the healthy control subjects (16/26 vs 53/65, $P = .04$). Smoking during pregnancy had been significantly more common among the mothers of the infants hospitalized for RSV infection than among those of the healthy control subjects (7/26 vs 9/84, $P = .03$), and smoking in the family at the time of the infection was more common than among the children with some other viral infection (13/26 vs 6/28, $P = .03$; Table I). No statistically significant differences between the groups were found in the other background variables. None of the participants had attended day care outside the home before the age of 6 months. Birth weight, birth order, smoking during pregnancy, and breast-feeding status at the time of entering the study were controlled for as confounding factors in the logistic regression analyses. For those with infection, we also controlled for the age at the time of the infection. In addition, smoking habits in the family were treated as a confounding factor when comparing the children hospitalized for RSV infection with the control subjects having some other viral infection.

Approximately half of the cord blood samples had a sufficient number of cells for the control and LPS stimulation experiments (Fig 1). The birth weight was significantly higher among the healthy control subjects with a successful analysis of unstimulated responses compared with those without (3656 g [SD, 549 g] vs 3384 g [SD, 412 g], $P = .032$). No other differences were found in the background factors when comparing those with successful analyses and those without.

Cytokine responses in LPS-stimulated cord blood cells

LPS-stimulated CBMCs from the subjects with RSV infection produced higher levels of IL-6 than those of the control subjects with another viral infection (31.0 ± 57.8 vs 1.1 ± 8.0 pg/mL, $P = .009$). Likewise, the IL-6 responses were significantly higher among the children hospitalized for RSV infection than among the healthy control subjects or the control subjects with another viral infection but remained at a lower level among the children treated as outpatients (Fig 2). IFN-γ responses to LPS stimulation were lower among the children hospitalized for RSV infection than among the healthy control subjects, and the stimulation also suppressed IL-5 production, which had decreased significantly more among the subjects with RSV infection than in the healthy control subjects (−1.3 ± 3.1 vs −0.5 ± 8.9 pg/mL, $P = .03$). No significant differences between the groups were found in the production of other cytokines, and IL-10 was undetectable in all groups (data not shown).

Factor analysis of the LPS-stimulated cytokine responses showed the children hospitalized for RSV infection to have higher factor scores on the combined IL-6 and IL-8 responses (reflecting higher responses) than the healthy control subjects ($P = .02$) or the children treated for RSV infection as outpatients ($P = .005$), and high scores on the combined IL-6 and IL-8 responses in logistic regression analysis predicted severe RSV infection relative to the scores for the healthy control subjects (odds ratio, 2.20; 95% CI, 1.17-4.14; $P = .01$; see Table E1 in

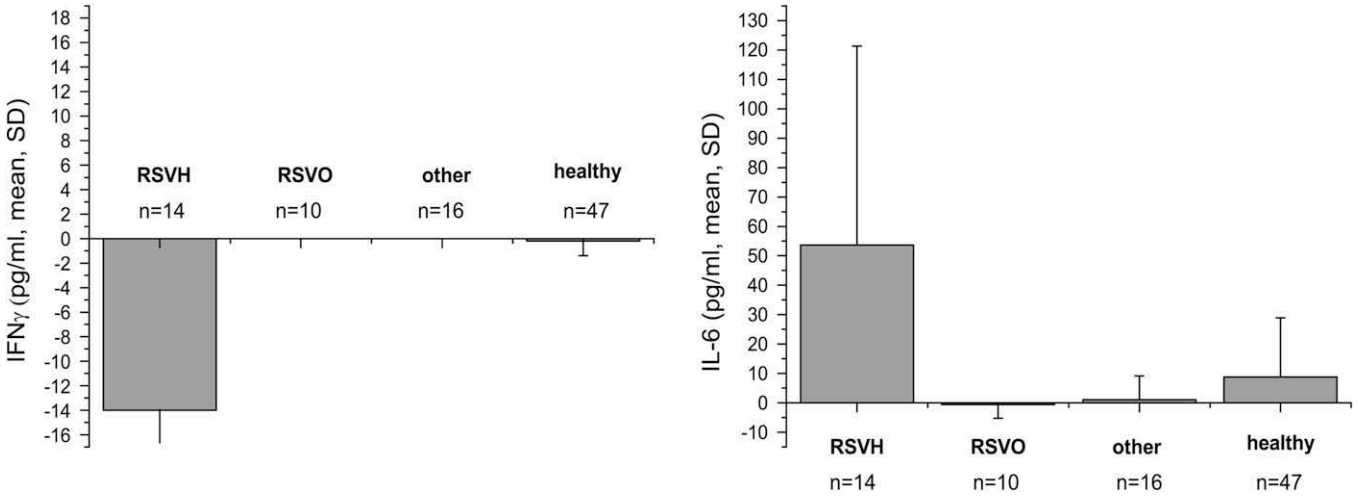

FIG 2. Cytokine responses in LPS-stimulated CBMCs, with significant differences between the groups. *RSVH,* Hospitalized patients with RSV; *RSVO,* patients with RSV treated as outpatients; *other,* control subjects with some other respiratory viral infection; *healthy,* healthy control subjects. IFN-γ: $P = .009$, RSVH versus healthy group. IL-6: $P < .001$, RSVH versus RSVO and *other* $P = .001$, RSVH versus healthy group.

this article's Online Repository at www.jacionline.org). The hospitalized children had lower scores on the combination of IL-1β, IL-2, IL-4, IL-5, and IL-10 responses than those treated as outpatients ($P = .02$), but this factor was not significantly related to RSV susceptibility or severity in the logistic regression analysis. The other factors, IFN-γ response alone and the combined responses of IL-12 and TNF-α, were not significantly associated with RSV infection.

Cytokine production in unstimulated CBMCs

The production of IL-6 and IL-12 from unstimulated CBMCs was significantly lower among the patients with RSV infection than among the healthy control subjects (3.5 ± 9.8 vs 7.4 ± 10.1 pg/mL [$P = .003$] and 5.6 ± 17.7 vs 11.7 ± 18.1 pg/mL [$P = .005$], respectively) and among those hospitalized for RSV infection than among the healthy control subjects (Fig 3), whereas IFN-γ and IL-4 responses were higher in children hospitalized for RSV infection than in the healthy control subjects. For IL-4, the difference between the hospitalized children and those with another viral infection was also significant. No significant differences between the groups were found in the production of IL-1β, IL-2, IL-5, IL-8, and TNF-α. IL-10 responses remained undetectable throughout except for those seen in 2 of the healthy control subjects (data not shown).

Factor analysis of cytokine responses in unstimulated cells from the healthy control subjects produced 4 factors that did not correlate with each other, all of which explained almost identical proportions of the total variance in cytokine responses (see Table E2 in this article's Online Repository at www.jacionline.org). The factors formed were the combined IL-6, IL-12, and TNF-α responses; the combined IL-2, IL-4, and IL-5 responses; the combined IFN-γ and IL-10 responses; and the combined IL-1β and IL-8 responses. Comparison of the factor scores derived from the loadings within each factor showed no differences between the patients with RSV infection, control subjects with another viral infection, and healthy control subjects, as analyzed by using the Student *t* test or logistic regression analysis.

DISCUSSION

Children with severe RSV infections during early infancy were seen here to have distinct LPS-stimulated cytokine response profiles already at birth, as shown by their higher combined IL-6 and IL-8 responses, which were found to predict severe infection in logistic regression analysis. The infants with a severe RSV infection had lower combined responses of IL-1β, IL-2, IL-4, IL-5, and IL-10 than those treated as outpatients, although this factor did not have any significant predictive value in logistic regression analysis.

A significant positive association between RSV infection and the later development of recurrent wheezing and asthma was found in a recent review of 12 selected published studies, but because of the limited methodological quality of the studies, the authors concluded that no reliable assumption of a possible causal relationship could be made.[4] We have previously shown that children with an early severe RSV infection have asthma more often and earlier than those who remain healthy during the same epidemic.[14] Likewise, children who had had an early RSV infection were found to differ from their healthy control subjects in their immune parameters 7 to 10 years later.[7] At the population level, children not exposed to an RSV epidemic by the age of 6 months were shown to develop asthma just as often as those exposed to such an epidemic, as evaluated by the later consumption of asthma medication.[8] Now we see that children with an early severe RSV infection differ from their healthy counterparts in their LPS-induced cytokine responses because they already have a higher capacity to produce the proinflammatory cytokines IL-6 and IL-8 at birth. These findings suggest that the children who are sensitive to RSV infection are those with an inborn increased risk of asthma and wheezing and that the RSV infection in itself does not necessarily induce the later development of asthma. Because of the heterogeneity of asthma, no direct comparisons between our findings and the cytokine responses found in asthmatic children can be made. The timing of birth has been suggested to affect both the susceptibility to an early RSV infection and asthma at 5 years' follow-up.[15] It has further been shown among children with a high risk of atopy that an early wheezing

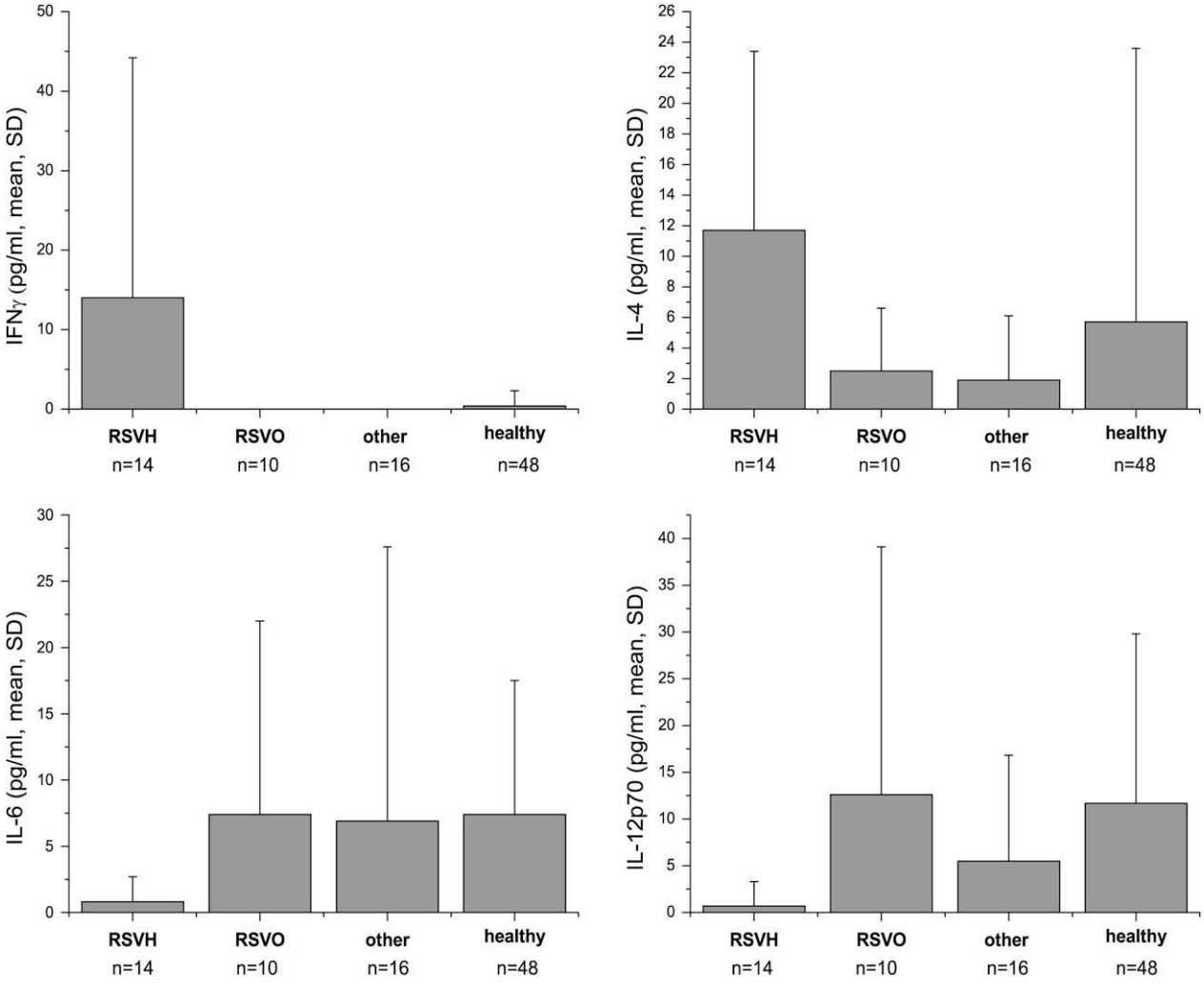

FIG 3. Cytokine responses in unstimulated CBMCs, with significant differences between the groups. *RSVH*, hospitalized patients with RSV; *RSVO*, patients with RSV treated as outpatients; *other*, control subjects with some other respiratory viral infection; *healthy*, healthy control subjects. IFN-γ: $P = .03$, RSVH versus healthy group. IL-4: $P = .03$, RSVH versus *other* $P = .01$, RSVH versus healthy group. IL-6: $P = .002$, RSVH versus healthy group. IL-12: $P = .003$, RSVH versus healthy group.

lower respiratory tract infection predicted wheezing at the age of 5 years, but statistical significance for current asthma was found only among those with an early rhinovirus infection and not with an RSV infection.[16] In the present study we compared groups of children born at the same time of the year and showed that there was a subgroup of children with susceptibility to a severe infection. We wanted the children to be born outside an RSV season but to be less than 6 months of age during the forthcoming RSV season because it has been suggested that the possible effect of an RSV infection on the development of asthma would be strongest at this age. We are not aware of studies showing differences in innate immune responses between infants born during different times of the year. The development of asthma has not been studied among our study children, but our previous studies have suggested that in the long run children with an early exposure to an RSV epidemic do not differ in this sense from those with later exposure.[8]

PHA-induced IL-13 responses have been shown to be lower at birth among those with wheezing during RSV infection than in those with no RSV infection, whereas children with detectable RSV-induced IFN-γ responses are less likely to wheeze during infections.[17] A low concentration of IL-12 in the serum of cord blood has also been found to be associated with subsequent bronchiolitis.[18] Because of the overlapping functions of cytokines and their complex regulatory network, it is clear that no one cytokine alone is responsible for the variation in susceptibility to an infection or in its severity. Interactions and overlapping effects of cytokines complicate statistical analyses and might result in biologically unrelated statistical associations. To resolve these problems, we used factor analysis to combine the responses that were closely intercorrelated, thus forming new variables that could be used in further analyses. In the factor analysis we used the cytokine assay data of the healthy control subjects only to describe their cytokine profiles and then to compare the factor scores with those with an infection. Another way to perform the analysis would have been merely to describe the cytokine profiles of each group separately, but in that case statistical comparisons between the groups would have been more complicated.

CBMCs have been shown to produce high amounts of IL-6 in response to LPS stimulation or RSV infection.[19-21] We found that LPS stimulation of cord blood cells resulted in higher IL-6 production levels in children with a severe RSV infection later in infancy. Although the activation of innate immunity and the production of proinflammatory cytokines are necessary for the elimination of a pathogen, an exaggerated immune response might contribute to the pathogenic sequelae of the infection. The main functions of the proinflammatory cytokines are to increase vascular permeability and endothelial cell adhesiveness, to recruit immune cells like neutrophils and monocytes to the site of inflammation, and to activate phagocytes and natural killer cells.[22] IL-6, with its proinflammatory properties, and IL-8, as a chemokine attracting and activating neutrophils, might influence the severity of RSV infection if produced in excess amounts.[23,24]

We studied only CBMCs. Studying the innate immune responses in alveolar mononuclear cells would be a more precise place to show immunologic differences affecting the severity of an infection. This kind of a study would, however, be impossible to perform when speaking of healthy children before any clinical disease appears. RSV infection itself has been shown to upregulate the production of several cytokines in the lungs. IL–6 and IL–8 expression have been shown to be higher in nasal washes, tracheal aspirates, and bronchoalveolar lavage samples of children with a severe RSV-induced bronchiolitis compared with samples from control subjects either with a non–RSV-induced bronchiolitis, with a milder RSV infection, or without a respiratory disease, although in one study the children hospitalized for an RSV infection did not differ from those treated as outpatients.[25-29] Little is known about cord blood immune responses as predictors for RSV infection, and virtually no other studies have been performed on the role of IL–6 and IL–8 responses in this respect. However, the genes of innate immunity have been shown to have the strongest association to RSV bronchiolitis.[30] During RSV infection, the plasma levels of IL–6 and IL–8 have been found to be higher in severely ill infants compared with levels seen in those with a milder infection and healthy control subjects.[31-33]

In line with our findings, it has been suggested that *IL8* gene polymorphism might be associated with the severity of RSV infection and with postinfectious wheezing.[34,35] Increased expression of proinflammatory cytokines, such as IL–1β, IL–6, and TNF–α, and of chemokines has also been found in the lungs of asthmatic subjects.[36] Thus far, we have not studied the relation of cord blood cytokine responses to the development of asthma and atopy in these children.

Because participation in the study was based on parental interest to contact us (except for the healthy control subjects, who were personally invited), we did not catch all those children with a mild infection caused by RSV or other viruses. However, the number of children hospitalized for an RSV infection was close to that estimated from previous studies, namely 2.4% of all those with a cord blood sample taken.[37] There were significant differences between the groups in the frequency of smoking by mothers or family members, birth order, and the continuation of breastfeeding at the time of the infection. These factors, which might contribute to the cytokine responses of cord blood cells, susceptibility to a severe RSV infection, or both among otherwise healthy children born at term, were adjusted for in the logistic regression analyses of the factor scores.[38-42] Unfortunately, the number of cells in the cord blood samples was sufficiently high for stimulation in only about half of the cases. This is a clear limitation of the study, but we were still able to find statistically significant differences between the groups.

Children with an RSV infection in early childhood have been shown to have an increased risk of asthma and recurrent wheezing.[1-4] On the basis of our data, we suggest that RSV itself does not necessarily induce the development of asthma and recurrent wheezing, but there are certain infants who have an inherent predisposition both to severe RSV infection and to postinfectious wheezing and asthma. This predisposition is associated with increased stimulated proinflammatory cytokine production that is already detectable at birth.

Key messages

- **Infants with a severe RSV infection differ from healthy children in their innate immunity cytokine profiles before the infection.**
- **The differences in innate immunity might affect both the susceptibility to a severe RSV infection and the development of asthma.**

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TABLE E1. Summary of factor analysis, including factor loadings, from a model of cytokine responses in the LPS-stimulated CBMCs of healthy control subjects

	Factor 1	Factor 2	Factor 3	Factor 4
Cytokine	IL-1β (0.775)	IL-6 (0.831)	IL-12 (0.898)	IFN-γ (0.975)
	IL-2 (0.789)	IL-8 (0.824)	TNF-α (0.682)	
	IL-4 (0.922)			
	IL-5 (0.791)			
	IL-10 (0.909)			
Total variance explained (%)	37.4	15.4	13.7	10.5

Standardized factor-score variables derived from the loadings were used in the logistic regression analyses.

TABLE E2. Summary of factor analysis, including factor loadings, from a model of cytokine responses in the unstimulated CBMCs of healthy control subjects

	Factor 1	Factor 2	Factor 3	Factor 4
Cytokine	IL-6 (0.903)	IL-2 (0.831)	IFN-γ (0.802)	IL-1β (0.942)
	IL-12 (0.969)	IL-4 (0.948)	IL-10 (0.922)	IL-8 (0.985)
	TNF-α (0.908)	IL-5 (0.655)		
Total variance explained (%)	28.1	21.0	20.9	20.0

Standardized factor-score variables derived from the loadings were used in the logistic regression analyses.