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Enhanced T helper 1 and 2 cytokine responses at birth associate with lower risk of middle ear infections in infancy

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Cytokines and respiratory infections

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**Enhanced T helper 1 and 2 cytokine responses at birth associate with lower risk of
middle ear infections in infancy**

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Abstract

Background: Respiratory tract infectious symptoms and illnesses are frequent during early childhood, but their risk factors, including the effect of early immune regulation, are less known. Aim of the study was to analyse whether stimulated cord blood cytokine production

is associated with the frequency of respiratory tract infectious symptoms or infections during the first year of life.

Methods: The study population consisted of children of mothers from farm or non-farm rural environment from Austria, Finland, Germany and Switzerland who participated in a prospective birth cohort study (PASTURE: Protection against Allergy – study in Rural Environments) (N=550). Cord blood samples were stimulated with the combination of phorbol ester and ionomycin (P/I) for 24 hours and the production of IL-5, IL-10, TNF- α and IFN- γ was determined by using ELISA. Information about infectious morbidity was collected using weekly diaries.

Results: P/I stimulated production of IL-5 (adjusted risk ratio (aRR) for $\leq$ median production, 0.37; 95% Confidence Interval (CI), 0.25-0.55, aRR for $>$ median production, 0.41; 95%CI, 0.27-0.61 vs. production $<$ detection limit) and IFN- γ (aRR for $\leq$ median production, 0.62; 95%CI, 0.40-0.95, aRR for $>$ median production, 0.39; 95% CI, 0.25-0.62 vs. production $<$ detection limit) in cord blood were associated with lower number of weeks with reported middle ear infection. There was a tendency towards positive association with P/I stimulated TNF- α production and middle ear infections.

Conclusions: Our results suggest that increased Th2- and Th1-associated cytokine responses at birth may provide protection from later middle ear infections.

Key words: Cord blood, Cytokines, Interferon- γ , Interleukin-5, Interleukin-10, Middle ear infection, Respiratory infections, Respiratory symptoms, Tumor-necrosis-factor- α

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INTRODUCTION

Respiratory infectious symptoms and illnesses are frequent during early childhood. They cause significant stress to families and financial burden on whole society (1). Hence, it has been of interest to disentangle factors that may affect the incidence of these symptoms and infections. There are various known early life risk factors for respiratory tract infections, such as early entrance into day care (2, 3), older siblings (2, 4, 5), parental smoking (6, 7) and lack of breastfeeding (4, 8, 9). In contrast, early life use of raw cow's milk has been shown to reduce the risk of respiratory infections (10). Moreover, recent studies have shown that not only postnatal but also prenatal conditions affect the frequency and nature of respiratory symptoms and infections. For example, maternal smoking during pregnancy (4, 7) has been associated with an increased risk of respiratory symptoms during childhood. This is of interest, since smoking during pregnancy has also been linked with impaired neonatal toll-like-receptor-mediated immune responses (11). There are also studies reporting the effect of prenatal exposure to various environmental toxins (e.g. polychlorinated biphenyls, dioxins and organochlorines) on the frequency of respiratory infection in early life (12, 13).

To better understand how risk factors affect the development of infections in early life, it is important to identify early immune mechanisms that predict later manifestation of respiratory symptoms. One approach is to study systemic cytokine responses during early immune maturation. In previous studies, cytokine production in cord blood (CB) has been associated with the course and number of respiratory infectious symptoms, or symptomatic respiratory infections in early life (14-17). For example, high LPS-stimulated CB interleukin (IL) -6 and IL-8 and lower IL-1 β , IL-2, IL-4, IL-5 and IL-10 responses have predicted the severity of respiratory syncytial virus (RSV) infection during infancy (17). However, many of the previous studies have primarily concentrated on examining the association between cytokines

and allergic respiratory symptoms (18, 19) or only included high risk (*i.e* allergic parents) children (15, 16, 20) whereas information on whether the associations are similar with infectious diseases is lacking.

In this study, we therefore analysed associations between the stimulated production of CB cytokines and the frequency of respiratory symptoms and infections during the first year of life.

METHODS

Study population

The original study population consisted of participants in a prospective birth cohort study PASTURE (Protection against allergy in rural environments), which is an ongoing international study in five European countries: Austria, Finland, France, Germany and Switzerland. The study population consists of 1133 farm and non-farm children whose mothers were followed from the third trimester of pregnancy. The follow-up protocol has been described in more detail earlier (21-23). The French study population (n=203) did not contribute to the cytokine data and was excluded from the analysis. After the final exclusions, the total study population consisted of 550 children all born between September 2002 and May 2005 (see exclusion criteria below).

Diary data and outcome definitions

The parents filled in diary questionnaires including questions on infectious symptoms and health care attendance of their child between 9th and 52nd postnatal week. Parents were asked if their child had been “hale and hearty” during the previous seven days. If the child had not been completely healthy, the parents also filled in a questionnaire on different infectious

diseases and symptoms (i.e. cough, wheezing, rhinitis, fever ≥ 38.5 °C, middle ear infection, diarrhea, urinary tract infection, itchy rash, or some other illness) during the last seven days.

We used the following outcome definitions: number of non-healthy weeks and weeks with cough, rhinitis, fever, middle ear infection, or respiratory tract infection (RTI). RTI was defined as rhinitis or cough in the absence of wheeze during the same week. Cough was defined as cough without wheeze during the same week in order to minimize confounding by allergy. Middle ear infection as reported by parents was included in the analyses as a common infection coexisting with RTI.

Cord blood cytokine stimulation

Cytokine levels were measured from CB. Heparinized CB samples were stimulated for 24 and 48 hours with Staphylococcal enterotoxin B (SEB; final concentration 100 ng/ml), lipopolysaccharide (LPS; final concentration 100 ng/ml) or the combination of phorbol 12-myristate 13-acetate (5 ng/ml) and ionomycin (P/I; 1 µg/ml; all from Sigma Chemicals, St. Louis, Mo., USA, except LPS from Research Center Borstel, Borstel, Germany). Stimulated production of IL-5, IL-10, tumor-necrosis-factor- α (TNF- α) and interferon- γ (IFN- γ) was determined using enzyme immunoassay (sandwich ELISA; OptEIA™ Human ELISA set, BD Biosciences, San Diego, CA, USA) in central laboratory (Marburg, Germany). Assays were performed according to the manufacturer's instructions except a reduced reaction volume (50 µL in 384-well plate) and a higher concentrated first standard of 1000 pg/mL, respectively. Samples were run undiluted, in a 1:20 dilution or in a 1:40 dilution for the optimal detection of cytokine concentration. Detection limits (DLs) were 10.0 pg/ml for IFN- γ , 7.7 pg/ml for TNF- α , 8.0 pg/ml for IL-5 and 11.4 pg/ml for IL-10. Cytokine concentrations were standardized on the individual white blood cell count (pg per 10^6 white blood cells).

Contamination of cord blood with maternal serum was excluded by several means of analyses, including the absence of IgA antibodies, as reported earlier (24).

The percentage of samples above the DLs ranged from 43.4 to 80.9 for P/I, from 4.2 to 92.5 for LPS and from 7.5 to 76.8 for SEB. As reported earlier (23), the sufficient concentrations of all measured cytokines were best obtained following P/I stimulation, thus the other stimulations were excluded from the final analyses. The correlations between cytokines measured at 24 h and 48 h time points were generally high (Table 1), but the percentages of detectable cytokine values were in general higher after 24 h stimulation. Therefore, 24 h time point was selected for further analyses. Final analyses reported included P/I-stimulated IL-5, IL-10, TNF- α and IFN- γ (24 h) which were divided in three categories: below DL, below cohort-specific median of detected level ($\leq$ median), and above cohort-specific median ($>$ median; Online Supplement, Table A).

Exclusion criteria

Children with less than half the number of possible diary entries (<23 weeks) available ($n=40$) and/or no cytokine data available ($n=279$) were excluded from further analyses. If information on health, respiratory symptoms, and infections were missing from a diary, the corresponding week was excluded from the analyses. If data for a cytokine was missing, the child was excluded from the analysis of that cytokine. The same was done with other missing data. Thus, the total number of children in the adjusted analyses varied between 496 and 550.

Statistical analysis

Spearman's correlation coefficient was used to analyze correlation between cytokine measurements in 24 and 48 h time points. Poisson regression analysis was used to investigate relationship between the number of healthy weeks and weeks with respiratory symptoms or infections during the follow-up and various predictive factors. In the multivariate regression models, potential confounding variables were selected a priori based on biological plausibility and the data was collected from questionnaires during pregnancy and early childhood. These variables included: gender, living on farm, siblings, day care attendance, breastfeeding, maternal smoking, maternal history of asthma or allergic diseases, gestational weeks at birth, season of birth, mode of delivery and cohort (Table 2). The Poisson regression analyses were first adjusted only for cohort and living on farm (partially adjusted), and finally for all above mentioned variables (fully adjusted). Effect-modification was analyzed by adding "cohort $\times$ cytokine" -interaction term in the multivariable model, and if the interaction term had p -value <0.2 , the results for each cohort were presented separately. Sensitivity analyses were done using only the data from children with ≥ 40 reported weeks and using robust variance estimation. Analysis on healthy weeks was also done with logistic regression using "being healthy more than 75% of the reported weeks" as outcome. Finally, the relationship between middle ear infection (yes vs. no during the study period) and various predictive factors was investigated with logistic regression, including analysis for the whole study population and for different cohorts separately. The results are expressed as frequency distributions, adjusted relative risks (aRR) or adjusted odds ratios (aOR) and their 95% confidence intervals (95% CI). Data analysis was performed using IBM SPSS version 23.0 (IBM Corp., Armonk, NY, USA).

The parents of the studied children gave their written informed consent. The research protocol was approved by the local ethics committees of the participating centers.

RESULTS

Basic characteristics of the study children are shown in Table 2. The mean number of followed weeks was 42.1 per child, ranging from 23 to 44, with 48.2% of the families filling in all 44 diary questionnaires.

The median number of non-healthy weeks during the study period was 8 (Table 3). The most frequent symptom was rhinitis (92.2%), followed by cough (76.7%) and fever (70.9%; Table 3). Above median production of TNF- α was associated with higher number of non-healthy weeks in the fully adjusted model (aRR 1.19, 95%CI 1.00-1.42; Table 4). Above median production of IFN- γ was inversely associated with the number of non-healthy weeks (aRR 0.85, 95%CI 0.73-0.99) and the frequency of fever in the fully adjusted models (aRR 0.74, 95%CI 0.59-0.94; Table 4). The productions of IL-5 and IFN- γ were associated with lower number of weeks with reported middle ear infection. In contrast, the production of TNF- α was associated with increased number of weeks with middle ear infection. Production of IL-5 was associated with more frequent cough. The production of cytokines after P/I stimulation was not associated with the frequency of respiratory tract infection or rhinitis (Tables 4 and B).

In total, 354 (64.4%) out of 550 children had been healthy $\geq 75\%$ of the reported weeks. Results obtained with logistic regression using this outcome were closely similar to those of Poisson regression using the variable “non-healthy weeks” (results not shown). The final adjusted analyses were repeated with children having ≥ 40 reported weeks and by using robust

variance estimation, and the associations did not change (Online Supplement, Tables C and D). Some of the associations also differed between cohorts (Online Supplement, Tables E and F).

DISCUSSION

Since respiratory tract infectious symptoms and illnesses are frequent during early childhood, it is important to better understand the early immune mechanisms that predict manifestation of these symptoms or infections. Our study shows that increased Th2- and Th1-associated cytokine responses at birth, namely P/I stimulated IL-5 and IFN- γ , predicted a lower number of middle ear infection episodes during the first year of life. This suggests that early regulation of adaptive immune responses may provide protection from later infections. These results may have an impact on the understanding of the prevention of infections, but further studies are needed. One question is, whether the cytokine response is causally related to the frequency of these symptoms and infections, or a proxy of another factor influencing it.

Increased IFN- γ mRNA expression in CB monocytes in response to RSV has been associated with decreased risk of upper respiratory tract and middle ear infections (25). Moreover, increased CB mononuclear cell IFN- γ responses have been associated with lower number of viral respiratory infections after phytohemagglutinin (PHA) stimulation (14), and reduced risk of acute lower respiratory illness after *Blatella germanica* 2 -allergen stimulation (26) in the first year. In our study, the children with high production of IFN- γ after P/I stimulation had less often fever than the children with the lowest production. Among respiratory symptoms and infections, the association was only seen with the production of IFN- γ and lower number of middle ear infection episodes. IFN- γ has antiviral, immunomodulatory and – stimulatory effects and thus its role in early immune maturation and the maintenance of

healthy immune defence is unquestionable. The ability of leukocytes to react early at birth to P/I stimulation might be genetic and be related to specific high-producing IFN- γ genotype. Further studies are needed to identify prenatal factors, which may increase the capacity of leukocytes to produce IFN- γ already at birth.

Previous studies have shown that middle ear infection is more common in children having higher TNF- α production genotypes (27-29), although one study showed no association between TNF- α polymorphisms and risk for middle ear infection among Finnish children (30). In our study, the production of TNF- α was associated with increased risk of middle ear infection. Alper and associates also showed that middle ear infection episodes coincident with evidence of RSV and rhinovirus were more frequent in 1-9.3-year-old children with high production IL-10 phenotypes (29). However, we did not find associations between the P/I stimulated levels of IL-10 and the reported frequency of middle ear infection during the first year of life.

Zhang and associates reported that low IL-10/high IL-5 T-cell response after PHA or SEB stimulation was associated with susceptibility to acute respiratory infections compared with a more resistant high IL-10/low IL-5 phenotype with children at high-risk for atopy (20). Fernandes et al. found a positive correlation between granulocyte/macrophage-colony-stimulating factor: IL-5 responsive colony forming unit and frequency of acute respiratory infection with fever in the first year of life (31). On the other hand, Friedlander et al. found no correlation between symptomatic viral infections during infancy and PHA-induced IL-5 responses from CB (15). In one Finnish study infants hospitalized for RSV infection had lower IL-5 responses in CB than those treated as outpatients (17). In our study, IL-10 levels after P/I stimulation were not associated with the frequency of respiratory tract infections.

Production of IL-5 at birth showed protection against middle ear infection during the first year of life, but on the contrary, it was positively associated with the higher risk of cough. IL-5 is produced by T-helper 2 –type cells and has been linked to immunological mechanisms of allergic illnesses (15).

Some differences between the countries were seen in the associations between cytokine levels and respiratory tract infectious morbidity. These might be partly explained by differences in the living environment. The surroundings at the farms or at home might differ between countries and it has been shown that moisture damage in home associates with systemic inflammatory markers in children (32). There could be some differences in the use of health care facilities, which could affect the frequency of some symptoms or infections. The number of children with a particular respiratory tract symptoms or infections in each cohort may vary; some are rare and the effect of one infected child might be high in subpopulation analyses.

When comparing the results of this study and previous reports, several factors need to be taken into account. First, many papers have reported associations between cytokine polymorphism and different respiratory infections or symptoms (27-30). The comparison of these studies and our results is difficult, since we measured stimulated cytokine production of CB leukocytes. Also many environmental factors related to pregnancy or birth can affect cytokine levels. Second, methodological factors have an important influence on the results, since cytokine production depends on the stimulant used and the sample type (14-16, 26). Third, in many pediatric studies, the study population is highly selected consisting of children with high risk for allergic diseases (15, 16, 18, 20, 25) or children from different living environments (18, 25).

There are several strengths in our study. The study cohort was carefully followed up from pregnancy onwards, and the data on respiratory infectious symptoms and infections were prospectively collected by weekly diaries. On the other hand, no objectively collected examination based data was available. The study population consists of children living in rural environment hence the results may not be generalizable for children living in urban surroundings.

In conclusion, our results suggest that the functional status of the adaptive immunity differs already at birth between the children who will or will not develop symptoms of respiratory infections during the first year of life. These results add to the previous knowledge on associations between early immune responses and the development of respiratory infectious symptoms and illnesses in infancy.

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Table 1. Production of cytokines after P/I stimulation in two follow-up points in cord blood samples

	24 hour time point			48 hour time point			Spearman correlation
	Total* N	Samples within detection limits % (N)	Median† (25/75 percentiles)	Total* N	Samples within detection limits % (N)	Median† (25/75 percentiles)	
IL-5							
control	570	0 (0)	0 (0/0)	561	0 (0)	0 (0/0)	
P/I	570	65.8 (375)	13.2 (0/28.4)	559	72.6 (406)	25.5 (0/54.5)	0.842
IL-10							
control	542	0 (0)	0 (0/0)	529	0 (0)	0 (0/0)	
P/I	540	57.2 (309)	7.75 (0/19.9)	525	43.4 (228)	0 (0/12.9)	0.695
IFN-γ							
control	554	0 (0)	0 (0/0)	545	0 (0)	0 (0/0)	
P/I	543	73.8 (401)	671.4 (0/2836.3)	536	72.8 (390)	652.6 (0/3110.3)	0.825
TNF-α							
control	517	0 (0)	0 (0/0)	515	0 (0)	0 (0/0)	
P/I	512	80.9 (414)	619.4 (72.3/1735.51)	510	72.9 (372)	393.8 (0/1482.93)	0.786

Spearman correlations for the stimulated production of cytokines on 24 h and 48 h time points

*the originally included study population before exclusions due missing information on variables selected for final multivariable analyses

†values are pg / 10⁶ white blood cells

Table 2. Basic characteristics of the study population (N=550)

	n	%
Female	267	48.5
Gestational weeks at birth		
35-39	165	30.0
40	172	31.3
41-43	213	38.7
Season of birth		
Winter: December to February	143	26.0
Spring: March to May	164	29.8
Summer: June to August	113	20.5
Autumn: September to November	130	23.6
Mode of delivery		
Vaginal	461	83.8
Cesarean section	89	16.2
At least one older sibling	346	62.9
Breastfeeding		
No or <9.0 wks	89	16.2
9.0-26.0 wks	122	22.2
>26.0 wks	339	61.6
Day care attendance		
No	481	87.5
From 0-9.0 mo age	45	8.2
From 9.1-11.5 mo age	24	4.4
Maternal history of asthma or allergy*	121	22.0
Maternal smoking†	30	5.5
Living on farm	267	48.5
Cohort		
Austria	97	17.6
Switzerland	151	27.5
Germany	127	23.1
Finland	175	31.8

*doctor diagnosed asthma, atopic dermatitis and/or allergic rhinitis ever

†at the beginning of the diary follow-up

Table 3. Respiratory infectious symptoms and infections reported in the weekly diaries

		At least one week with symptom or infection	Number of weeks with symptom or infection
	N	n (%)	Median (25th/75th percentile)
Non-healthy	550	543 (98.7)	8 (5/13)
Respiratory tract infection	550	512 (93.1)	5 (2/8)
Rhinitis	550	507 (92.2)	4 (2/8)
Cough	550	422 (76.7)	2 (1/4)
Fever	550	390 (70.9)	1 (0/2)
Middle ear infection	550	119 (21.6)	0 (0/0)

Table 4. Results of the fully adjusted models for the associations between cord blood cytokine production and number of non-healthy weeks and weeks with respiratory symptoms or infections

	Total n	Non-healthy aRR (95% CI)	Respiratory tract infection aRR (95% CI)	Rhinitis aRR (95% CI)	Cough aRR (95% CI)	Fever aRR (95% CI)	Middle ear infection aRR (95% CI)
IL-5							
<detection limit	187	1	1	1	1	1	1
≤median	184	0.98 (0.84-1.13)	1.05 (0.88-1.23)	1.05 (0.89-1.25)	1.31 (1.03-1.65)	0.99 (0.80-1.24)	0.37 (0.25-0.55)
>median	179	1.04 (0.90-1.20)	1.12 (0.94-1.32)	1.11 (0.94-1.32)	1.34 (1.05-1.69)	0.94 (0.75-1.18)	0.41 (0.27-0.61)
<i>p</i> for interaction-term		.88	.50	.15	.99	.36	.29
IL-10							
<detection limit	221	1	1	1	1	1	1
≤median	149	1.09 (0.95-1.24)	1.06 (0.91-1.24)	1.02 (0.87-1.20)	1.13 (0.91-1.40)	0.84 (0.68-1.05)	0.73 (0.49-1.08)
>median	149	1.06 (0.92-1.21)	1.00 (0.85-1.18)	1.07 (0.92-1.26)	0.96 (0.76-1.20)	1.03 (0.84-1.27)	1.21 (0.84-1.73)
<i>p</i> for interaction-term		.43	.49	.64	.12	.59	.08
IFN-γ							
<detection limit	135	1	1	1	1	1	1
≤median	194	0.88 (0.76-1.02)	0.85 (0.71-1.01)	0.93 (0.78-1.11)	0.95 (0.74-1.21)	0.87 (0.70-1.09)	0.62 (0.40-0.95)
>median	195	0.85 (0.73-0.99)	0.90 (0.76-1.07)	0.98 (0.82-1.17)	0.97 (0.74-1.25)	0.74 (0.59-0.94)	0.39 (0.25-0.62)
<i>p</i> for interaction-term		.52	.38	.11	.70	.40	.002
TNF-α							
<detection limit	92	1	1	1	1	1	1
≤median	204	1.09 (0.92-1.30)	1.04 (0.85-1.28)	1.19 (0.90-1.36)	1.00 (0.75-1.32)	1.13 (0.86-1.48)	1.94 (1.04-3.63)
>median	200	1.19 (1.00-1.42)	1.16 (0.95-1.42)	1.18 (0.96-1.44)	1.26 (0.96-1.66)	1.01 (0.77-1.33)	1.32 (0.69-2.52)
<i>p</i> for interaction-term		.51	.28	.47	.57	.59	.64

Models are adjusted for gender, season of birth, living on farm, gestational weeks at birth, mode of delivery, siblings, breastfeeding, day care attendance, maternal smoking, maternal history of asthma or allergic diseases and cohort

Risk ratios are obtained by Poisson regression, bolded values have $p < .05$

P-values are for "cohort x cytokine" –interaction-term. The results for each cohort are presented separately in Online Supplement Table E for analyses with $p < .2$ (*bolded*)

Cytokine concentrations were measured following 24 h stimulation with the combination of phorbol ester and ionomycin and standardized on individual leukocyte counts

Cohort-specific medians were used for categorization of the cytokines