

Children with asthma by school age display aberrant immune responses to pathogenic airway bacteria as infants

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Background: Asthma is a highly prevalent chronic lung disease that commonly originates in early childhood. Colonization of neonatal airways with the pathogenic bacterial strains

Haemophilus influenzae, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* is associated with increased risk of later childhood asthma. We hypothesized that children with asthma have an abnormal immune response to pathogenic bacteria in infancy.

Objective: We aimed to assess the bacterial immune response in asymptomatic infants and the association with later development of asthma by age 7 years.

Methods: The Copenhagen Prospective Studies on Asthma in Childhood birth cohort was followed prospectively, and asthma was diagnosed at age 7 years. The immune response to *H influenzae*, *M catarrhalis*, and *S pneumoniae* was analyzed in 292 infants using PBMCs isolated and stored since the age of 6 months. The immune response was assessed based on the pattern of cytokines produced and T-cell activation.

Results: The immune response to pathogenic bacteria was different in infants with asthma by 7 years of age ($P = .0007$). In particular, prospective asthmatic subjects had aberrant production of IL-5 ($P = .008$), IL-13 ($P = .057$), IL-17 ($P = .001$), and IL-10 ($P = .028$), whereas there were no differences in T-cell activation or peripheral T-cell composition.

Conclusions: Children with asthma by school age exhibited an aberrant immune response to pathogenic bacteria in infancy. We propose that an abnormal immune response to pathogenic bacteria colonizing the airways in early life might lead to chronic airway inflammation and childhood asthma. (J Allergy Clin Immunol 2014;133:1008-13.)

Key words: Childhood asthma, bacteria, immune response, birth cohort

Asthma is a complex disease representing several endotypes with different genetic risk factors and environmental triggers.¹ Chronic airway inflammation is a central hallmark of asthma, and many of the known risk genes are associated with immune function.²

The Copenhagen Prospective Studies on Asthma in Childhood (COPSAC₂₀₀₀) birth cohort showed that colonization of the airways in 1-month-old asymptomatic neonates by specific pathogenic bacteria (*Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*) was associated with increased risk of asthma at 5 years of age, suggesting that the human microbiome is an important environmental trigger of chronic inflammation in susceptible subjects.³

We tested the hypothesis that children with later asthma had an abnormal immune response to pathogenic bacteria in infancy. We speculated that a deficient bacterial immune response could allow bacterial colonization caused by inadequate clearance by the immune system. In turn, persistent colonization might initiate the chronic airway inflammation characteristic of asthma in genetically predisposed infants.

Cytokines produced by the immune system perform specific roles in mediating antimicrobial mechanisms, including recruitment and activation of immune cells appropriate to eliminate a specific pathogen. However, dysfunctional immune responses are a hallmark of autoimmune and atopic disorders, including asthma.^{4,5} Storage of PBMCs harvested from the same COPSAC₂₀₀₀ birth cohort by 6 months of age has allowed us to interrogate the bacterial immune response in infancy before the development of asthma. We assessed the production of key cytokine immune mediators involved in bacterial defense (TNF- α , IFN- γ , and IL-17),⁶⁻⁹ asthma immunopathology (IL-5 and IL-13),¹⁰⁻¹² and immune regulation (IL-10 and IL-2)^{13,14} from these PBMCs when exposed to *H influenzae*, *M catarrhalis*, and *S pneumoniae*.

We report that infants who go on to have asthma have an aberrant immune response to colonizing pathogenic airway bacteria characterized by increased production of asthma-associated cytokines and inappropriate antibacterial properties. We hypothesize that an abnormal immune response to bacteria in the airways allows persistent colonization and represents a path to the chronic airway inflammation of childhood asthma.

METHODS

Study cohort

The study was part of the ongoing COPSAC₂₀₀₀ prospective birth cohort. This cohort consists of 411 children born to mothers with active or previous doctor-diagnosed asthma enrolled in the period 1998-2001. The study was

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Abbreviations used

COPSAC: Copenhagen Prospective Studies on Asthma in Childhood
FACS: Fluorescence-activated cell sorting
NK: Natural killer
PC: Principal component
PCA: Principal component analysis
TCR: T-cell receptor

approved by the Ethics Committee for Copenhagen (KF 01-289/96) and the Danish Data Protection Agency (2002-41-2434) and followed the principles of the Declaration of Helsinki. Written informed consent was obtained from the mothers at enrollment. Children enrolled in the COPSAC₂₀₀₀ birth cohort were followed prospectively by our clinical research team, with regular follow-up visits every 6 months and at episodes of significant acute respiratory symptoms in accordance with standard operating procedures published on our Web site. The families of enrolled children used our research unit doctors (not general practitioners) for diagnosis and treatment of any respiratory or atopy-related symptoms. Good clinical practice guidelines were followed for data validation and quality control. Clinical data were collected online during visits, and the database was double checked against source data by an external monitor and subsequently locked. An audit trail was run routinely.

Bacterial immune responses

Immune responses to pathogenic airway bacteria were studied in PBMCs. Four milliliters of peripheral blood was drawn at the 6-month routine visit to the clinical research unit. PBMCs were isolated by means of density centrifugation and stored at -140°C for up to 12 years until the cells were analyzed during 2011 (see the [Methods](#) section in this article's Online Repository at www.jacionline.org for details on PBMC preparation, bacterial preparation, culturing, and flow cytometry). PBMCs were stimulated with UV-inactivated *H influenzae*, *M catarrhalis*, or *S pneumoniae*, and immune response was assessed based on production of cytokines in culture supernatants and T-cell activation propensity. The supernatant cytokines IFN- γ , TNF- α , IL-17, IL-5, IL-13, IL-10, and IL-2 were analyzed by using a custom multiplex assay from Meso Scale Discovery and read on a Sector Imager 6000 (MSD, Gaithersburg, Md). Helper (CD4^{+}) and cytotoxic (CD8^{+}) T-cell activation abilities were analyzed by assessing the expression levels of OX40, CD25, and CD69 using flow cytometry. PBMC samples were analyzed in random order, and all stimulations, measurements, and data handling were performed in a blinded manner.

Immune phenotyping

Composition of the T-cell compartment of each child was assessed as helper ($\text{CD3}^{+}\text{CD4}^{+}$), cytotoxic ($\text{CD3}^{+}\text{CD8}^{+}$), regulatory ($\text{CD3}^{+}\text{CD4}^{+}\text{CD127}^{-}\text{CD25}^{+}$), $\gamma\delta$ (CD3^{+} T-cell receptor [TCR] $\gamma\delta^{+}$), and invariant natural killer (NK; $\text{CD3}^{+}\text{TCRV}\alpha 24\text{-J}\alpha 18^{+}$) T cells using flow cytometry with a predefined gating strategy. All populations were calculated relative to the CD3^{+} T-cell compartment for each donor (see the [Methods](#) section in this article's Online Repository for details on flow cytometry).

Asthma diagnosis

The primary end point was current asthma by 7 years of age analyzed as a dichotomized variable. Asthma was diagnosed, as previously described in detail.¹⁵⁻¹⁷ The diagnosis was based on a rigid algorithm requiring a history of recurrent significant troublesome lung symptoms documented in daily diary cards. Symptoms were judged by the study doctor to be typical of asthma (eg, exercise-induced symptoms, prolonged nocturnal cough, persistent cough outside common cold, and symptoms causing wakening at night). Response to a 3-month course of inhaled corticosteroids, relapse at withdrawals, and need

for intermittent use of an inhaled β_2 -agonist to relieve dyspnea were required for a confirmed diagnosis.

Statistical analysis, modeling, and data visualization

Measurements of supernatant cytokines and T-cell activation markers in response to *H influenzae*, *M catarrhalis*, or *S pneumoniae* were adjusted by subtracting baseline levels of unstimulated PBMCs for each donor. Measurements were subsequently square root transformed to improve normal distribution for statistical analysis. Multiple logistic regression analysis was used to test the association between each of the cytokines or T-cell activation markers analyzed in PBMC cultures and asthma at 7 years of age. The analysis included measurements of each variable from all 3 bacterial stimulations in relation to asthma outcome. The association of the peripheral T-cell phenotype at 6 months of age with asthma at age 7 years was analyzed for each T-cell population by using logistic regression. The presented odds ratios and 95% CIs are all univariate, and were calculated based on variables standardized by their SDs. Statistical analysis was performed with the SAS 9.3 software package (SAS Institute, Cary, NC).

Principal component analysis (PCA) modeling was used to decompose the complex data set into fewer dimensions to extract patterns that describe the greatest variations in the immune response to pathogenic airway bacteria. These patterns reflect both the systematic intercytokine correlation structure and how this relates to specific bacterial stimulation. For further details on the PCA decomposition, see the [Methods](#) section in this article's Online Repository.

The PCA model describing the bacterial immune response among all children in the cohort was used to analyze the association between the immune response in infancy and asthma development at age 7 years. Multivariate statistical inference was calculated by using a logistic regression on asthmatic versus nonasthmatic groups by including the first 4 principal components (PC1-PC4), describing most variation in the PCA model as independent variables. PCA analysis and visualization were performed with the MatLab 2011a version 7.12.0.635 software package (MathWorks, Natick, Mass) with the PLS toolbox version 6.5.1 (Eigenvector Research, Wenatchee, Wash).

RESULTS

Study cohort

PBMC collection and storage at age 6 months were successfully completed in 331 (81%) of the 411 infants enrolled in the COPSAC₂₀₀₀ cohort at birth. Two hundred ninety-two (88%) of the 331 children had information on asthma by age 7 years. The asthma prevalence was 13% (38 asthmatic vs 254 nonasthmatic subjects). No important differences between the study and dropout groups were observed (see baseline characteristics of the study groups in [Table E1](#) in this article's Online Repository at www.jacionline.org). [Table I](#) shows absolute cytokine and T-cell activation marker levels measured in response to bacterial challenge, and [Table II](#) shows the measured helper, cytotoxic, $\gamma\delta$, and invariant NK T-cell profiles in infants.

Bacterial immune responses in relation to asthma

The bacterial immune response to *H influenzae*, *M catarrhalis*, and *S pneumoniae* in infancy (6 months) was characterized by increased IL-5 ($P = .008$) and IL-13 ($P = .057$) levels to all 3 bacteria in infants with asthma by 7 years of age ([Table III](#)). The group of prospective asthmatic subjects also had increased IL-17 ($P = .001$) and IL-10 ($P = .028$) levels in response to Gram-negative *H influenzae* and *M catarrhalis*, whereas these levels were decreased when stimulated with Gram-positive *S pneumoniae*.

TABLE I. Measured cytokine and T-cell activation levels in response to pathogenic airway bacteria at age 6 months (n = 292)

	Control, median (IQR)	<i>H influenzae</i> , median (IQR)	<i>M catarrhalis</i> , median (IQR)	<i>S pneumoniae</i> , median (IQR)
Cytokine supernatant				
IFN- γ (pg/mL)	0.4 (0.0-1.1)	4.7 (0.3-77.9)	19.9 (0.9-239.5)	1.5 (0.2-13.1)
TNF- α (pg/mL)	1.5 (0.5-4.1)	34.7 (7.5-180.2)	55.5 (16.2-212.1)	21.3 (5.0-102.6)
IL-5 (pg/mL)	0.0 (0.0-0.0)	0.0 (0.0-0.4)	0.4 (0.0-2.4)	0.0 (0.0-0.6)
IL-13 (pg/mL)	0.0 (0.0-0.0)	14.8 (0.0-78.9)	57.6 (5.4-196.9)	11.0 (0.0-71.6)
IL-17 (pg/mL)	0.0 (0.0-1.8)	2.1 (0.0-13.1)	8.7 (0.4-44.0)	2.3 (0.0-17.9)
IL-10 (pg/mL)	0.0 (0.0-1.0)	14.8 (2.1-62.0)	22.0 (3.6-84.1)	2.1 (0.0-10.5)
IL-2 (pg/mL)	0.8 (0.0-4.1)	4.7 (1.1-14.2)	11.8 (3.1-28.6)	9.5 (1.3-41.8)
Helper T-cell activation				
OX40 ⁺ (%)	1.6 (1.1-2.3)	2.9 (1.9-4.1)	7.9 (4.9-11.7)	3.5 (2.3-5.3)
CD25 ⁺ (%)	8.7 (7.2-10.4)	11.1 (8.9-13.5)	17.6 (13.3-22.8)	10.7 (8.4-13.1)
CD69 ⁺ (%)	0.4 (0.2-0.6)	1.5 (0.6-2.6)	8.1 (4.9-11.6)	1.7 (0.9-2.6)
Cytotoxic T-cell activation				
CD25 ⁺ (%)	1.2 (0.9-1.8)	1.9 (1.3-2.9)	4.9 (3.0-7.3)	2.4 (1.7-3.7)
CD69 ⁺ (%)	0.3 (0.2-0.5)	1.0 (0.5-1.7)	4.4 (2.7-6.8)	1.9 (1.3-2.7)

IQR, Interquartile range.

IL-5 was undetectable in 53% of *H influenzae*–, 37% of *M catarrhalis*–, and 49% of *S pneumoniae*–stimulated samples. Thus to confirm increased IL-5 production as a risk factor for asthma development, we performed an analysis including only infants with detectable IL-5 levels for all 3 bacteria. The association between detectable IL-5 levels in the infant bacterial immune response and asthma at age 7 years was statistically significant ($P = .010$, $n = 109$, 17 asthmatic vs 92 nonasthmatic subjects).

To further characterize the bacterial immune response, we used multivariate PCA. This method reduces the complexity of the data set by means of extracting information from correlated variables (cytokines and bacteria) as so-called PCs. A PCA analysis was performed, including the measured cytokine profile in response to *H influenzae*, *M catarrhalis*, and *S pneumoniae*. The PCA analysis (Fig 1, A) revealed that the predominant variation in bacterial immune response within the cohort could be ascribed to (1) donor-specific variation in all cytokine levels (increased levels along PC1 explained 62% of the variation) and (2) individual bacteria used to challenge immune cells (cytokine discrimination along PC2 explained 15% of variation).

The PCA analysis was found to significantly describe the differences in bacterial immune responses between infants with or without asthma at age 7 years ($P = .0007$, multivariate analysis of PC1-PC4). This allows for interpretation based on the model obtained from the PCA analysis facilitated by means of visualizing derived PCs. The average immune response to each bacterium among prospective asthmatic and nonasthmatic subjects are depicted in the PCA analysis (Fig 1). PC2 and PC3 were found to best describe the differences in bacterial immune responses between prospective asthmatic and nonasthmatic infants (Fig 1, B). Specifically, the infant bacterial immune response in prospective asthmatic subjects at 7 years of age is associated with increased levels of type 2 cytokines (IL-5 and IL-13) and a relative decrease in levels of both the immune regulatory cytokine IL-10 and the type 1 cytokines (IFN- γ and TNF- α). It is observed that the cytokine profiles induced by the Gram-negative *H influenzae* and *M catarrhalis* are similar (Fig 1, B, overlapping confidence area and similar response pattern) and that both are different from the response to the Gram-positive *S pneumoniae* (response pattern perpendicular to Gram-negative bacteria).

TABLE II. Phenotypic composition of the T-cell compartment at 6 months of age (n = 242)

T-cell profile	Phenotype, median (IQR)*
Helper T cells	68.5 (62.6-72.4)
Cytotoxic T cells	24.1 (20.6-29.9)
Regulatory T cells	2.31 (1.79-2.91)
$\gamma\delta$ T cells	3.01 (2.23-3.87)
Invariant NK T cells	0.07 (0.05-0.09)

IQR, Interquartile range.

*Values are percentages of all (CD3⁺) T-cells.

Bacteria-induced T-cell activation and peripheral T-cell phenotype in relation to asthma

T-cell activation measured based on surface expression of OX40, CD25, and CD69 on helper and cytotoxic T cells in response to challenge with the 3 pathogenic airway bacteria showed no association with later development of asthma (Table III). Moreover, addition of the T-cell activation data set to the PCA model of cytokines did not add further distinction between prospective asthmatic and nonasthmatic subjects.

The composition of peripheral T-cell subsets (without stimulation with bacteria) at 6 months of age was not linked to the risk for asthma at 7 years of age (Table IV).

DISCUSSION

Main findings

Children with asthma by school age exhibited an abnormal immune response to pathogenic bacteria already at the age of 6 months. This aberrant immune response was characterized by increased IL-5 and IL-13 levels in response to all 3 pathogenic airway bacteria, whereas IL-17 and IL-10 levels were enhanced with Gram-negative *H influenzae* and *M catarrhalis* challenge and decreased for Gram-positive *S pneumoniae*. Neither activation of T cells (surface expression of OX40, CD25, and CD69) nor the composition of the peripheral T-cell compartment (helper, cytotoxic, regulatory, $\gamma\delta$, and invariant NK T cells) showed any association with later development of asthma. Such an aberrant cytokine response to pathogenic bacteria colonizing the airways

TABLE III. Bacterial immune responses at age 6 months and risk of asthma diagnosis at age 7 years (n = 292)

	<i>H influenzae</i> , OR (95% CI)	<i>M catarrhalis</i> , OR (95% CI)	<i>S pneumoniae</i> , OR (95% CI)	P value*
Cytokine supernatant				
IFN- γ	1.06 (0.77-1.47)	1.06 (0.76-1.47)	0.98 (0.69-1.38)	.905
TNF- α	1.12 (0.80-1.56)	1.09 (0.77-1.52)	0.89 (0.61-1.29)	.187
IL-5	1.69 (1.24-2.30)	1.37 (1.03-1.82)	1.17 (0.87-1.59)	.008
IL-13	1.40 (1.04-1.90)	1.24 (0.90-1.71)	1.22 (0.90-1.56)	.057
IL-17	1.14 (0.83-1.56)	1.32 (0.99-1.76)	0.81 (0.54-1.23)	.001
IL-10	1.09 (0.78-1.52)	1.24 (0.89-1.72)	0.89 (0.61-1.30)	.028
IL-2	1.14 (0.80-1.62)	1.05 (0.75-1.46)	1.09 (0.79-1.50)	.848
Helper T-cell activation				
OX40 ⁺	1.12 (0.80-1.56)	1.07 (0.77-1.48)	0.69 (0.47-1.03)	.098
CD25 ⁺	1.14 (0.82-1.59)	1.08 (0.78-1.51)	0.71 (0.50-1.01)	.066
CD69 ⁺	0.97 (0.69-1.35)	1.01 (0.72-1.40)	0.85 (0.60-1.21)	.757
Cytotoxic T-cell activation				
CD25 ⁺	1.14 (0.82-1.60)	1.16 (0.84-1.61)	0.86 (0.61-1.22)	.392
CD69 ⁺	1.00 (0.71-1.41)	1.10 (0.79-1.53)	0.79 (0.55-1.14)	.352

OR, Odds ratio.

*P value from multiple logistic model including measurements from all 3 bacteria.

in early life might lead to chronic airway inflammation and childhood asthma.

Strengths of the study

Storage of PBMCs from infancy in this prospective clinical birth cohort study of asthma development in childhood provided unique material for prospective analysis of infant immune function preceding disease development 7 years later.

Accuracy of the end point is a particular strength to the study because of the tight prospective clinical follow-up with deep phenotyping. The research clinic served as a health center for the children of the cohort who came regularly for 6-month visits and at episodes of acute respiratory symptoms to receive a diagnosis. Children were only managed by our research doctors with pediatric training according to standard operating procedures, and all families monitored lung symptoms in diaries throughout the follow-up period. This stringent methodology avoided inconsistent clinical practice among community doctors on the diagnosis and treatment of early childhood respiratory symptoms.

H influenzae, *M catarrhalis*, and *S pneumoniae* used for the assessment of bacterial immune responses are associated with respiratory infections and asthmatic exacerbations¹⁸ but are also common pathogens colonizing the airways of healthy newborns, which we previously demonstrated were associated with development of childhood asthma in our COPSAC₂₀₀₀ birth cohort.³ The PBMC immune response of the 6-month-old infants to these bacteria was characterized by a panel of T cell–associated cytokines in supernatants and surface expression of activation markers on T cells. The cytokines represent a broad panel of functional properties associated with T cells, including type 1 (IFN- γ and TNF- α), type 2 (IL-5 and IL-13), type 17 (IL-17), and regulatory (IL-10 and IL-2) T-cell responses. Cytokines were measured by using an electrochemiluminescence multiarray platform technology detecting low levels of proteins at a high precision. The panel of cytokines represents prototypic cytokines with well-characterized immune function and of importance in relation to both asthma immunopathology and bacterial defense.

We used PCA to reduce the complexity of analyzing 21 independent but immunologically highly interrelated variables (3 bacterial stimuli and 7 cytokines measured). Furthermore, the

PCA method allows us to extract and visualize covariation between the variables (cytokines and bacteria) that might be relevant in relation to the study outcome. The data analysis was conducted in a 2-step fashion. The first step involves an unsupervised overview of the main variations in the data set by using PCA. Here we interrogate bacterial differences and similarities together with the cytokine correlation structure. Second, because interpretation is of key interest, the obtained PCA model of the data set was used in a predictive fashion toward the end point of asthma at 7 years of age. Logistic regression analysis using the PCA model for predictors found the first 4 principal components to describe our outcome, thus allowing interpretations to be made on the covariation between bacteria and cytokines in relation to later development of asthma. The findings suggest that it is not the overall level of a single or a set of cytokines that discriminates future asthmatic and nonasthmatic subjects but rather a skewed immune profile between immune responses of different bacteria (principal component 2 and 3) and that this is to some extent independent of the overall immune response level registered in principal component 1. Because the PCA results are based on 12 independent variables and 292 subjects in the logistic regression analysis, our findings are unlikely to occur because of overfitting (type I statistical error).

The Gram-negative *H influenzae* and *M catarrhalis* induced similar immune responses that were different from the Gram-positive *S pneumoniae*. This species-specific bacterial immune response supports the specificity of our findings.

Limitations of the study

It is a study limitation that all children were born to mothers with an asthma history, and thus our findings require replication in unselected birth cohorts for external validity.

It is another study limitation that the immune response was assessed *in vitro* because it might not adequately reflect the complex *in vivo* processes involving tissue-resident immune cells.

Finally, it is a limitation to the interpretation of our data that we characterized the composition of the peripheral T-cell phenotypes in infants by analyzing the relative numbers of helper, cytotoxic, regulatory, $\gamma\delta$, and invariant NK T cells within the T cell compartment. Because the characterization was performed on

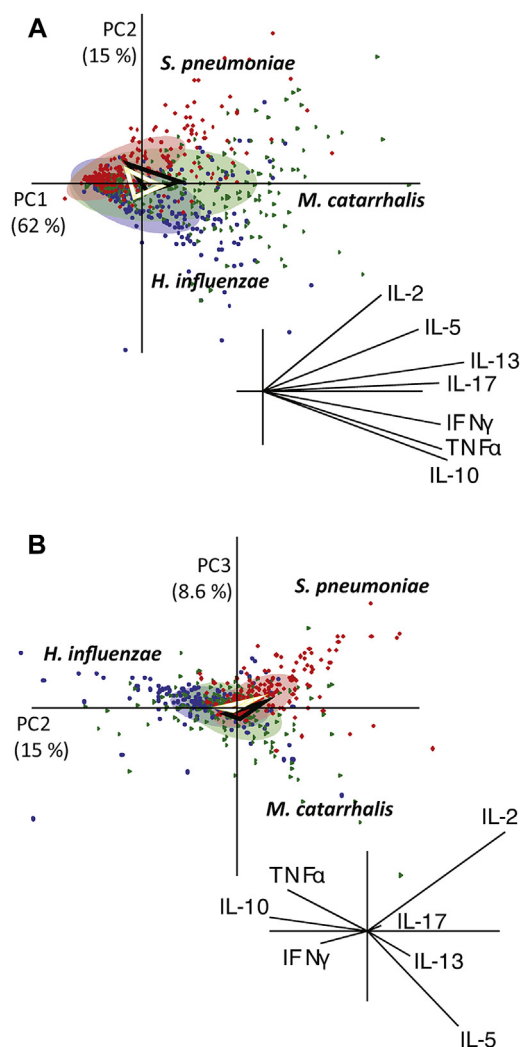


FIG 1. PCA of the immune response to pathogenic airway bacteria in 6-month-old infants and the relation to asthma diagnosis at 7 years of age. Infant immune responses to *H influenzae* (blue), *M catarrhalis* (green), and *S pneumoniae* (red) was assessed based on the production of the cytokines IFN- γ , TNF- α , IL-17, IL-5, IL-13, IL-10, and IL-2 in PBMC cultures and visualized by using PCA (PC1 vs PC2 [A] and PC2 vs PC3 [B]). Dots represent the combined cytokine response of an infant to one of the bacteria (3 dots per donor). Shaded areas represent the 67% confidence area in the cytokine response of all infants to each bacterium. Triangles show the average bacterial immune response in infancy among prospective asthmatic (black triangles, $n = 38$) and nonasthmatic (white triangles, $n = 254$) children at 7 years of age ($P = .0007$). Each corner of the triangle represents the average immune response to each bacterium within the group. Fig 1, A, The loading plot shows the overall increased cytokine levels after bacterial challenge (PC1) and the cytokine profiles was broadly segregated by bacterial specific response (PC2). Fig 1, B, PC3 in the loading plot further displays the cytokine patterns separating the individual bacteria.

PBMC samples, we were not able to determine the absolute concentration of cells in the blood of the infants. This is a weakness because the relative cell numbers might not reflect potential developmental differences in the T-cell compartment within the infants.

Interpretation

The aberrant immune response to pathogenic airway bacteria in infants developing asthma at 7 years of age is suggestive of an

TABLE IV. T-cell immune phenotype at age 6 months and risk of asthma diagnosis at age 7 years ($n = 242$)

T-cell profile	Phenotype, OR (95% CI)	P value
Helper T cells (%)	0.84 (0.55-1.28)	.425
Cytotoxic T cells (%)	1.17 (0.77-1.77)	.469
Regulatory T cells (%)	1.00 (0.65-1.55)	.996
$\gamma\delta$ T cells (%)	0.96 (0.61-1.49)	.845
Invariant NK T cells (%)	1.32 (0.89-1.93)	.163

OR, Odds ratio.

inability to clear these bacteria in the airways on exposure. Reduced bacterial clearance in future asthmatic subjects might permit persistent colonization, as recently suggested,¹⁹ which in turn could drive chronic inflammation and asthma.

The combination and levels of cytokines produced during an immune response determine the inflammatory processes initiated to eliminate a particular microorganism. The bacterial immune response in infants on a trajectory to asthma was skewed away from main production of type 1 cytokines (IFN- γ and TNF- α) that eliminate intracellular bacteria, such as Gram-negative *H influenzae* and *M catarrhalis*, through macrophage recruitment and activation and promotion of cytotoxic T-cell and NK cell function.^{6,7} IL-17 production, which is required for neutrophil activation in the eradication of extracellular bacteria, such as *S pneumoniae*,^{8,9} was selectively reduced in response to *S pneumoniae* in PBMCs of infants with later asthma. In addition to the reduced antibacterial properties, it is also intriguing that the immune response to all 3 bacteria in infants with later asthma was characterized by increased levels of type 2 cytokines (IL-5 and IL-13). These cytokines are central mediators of the asthma immunopathology causing eosinophil activation, mucus production, and airway hyperreactivity.⁴ The importance of these cytokines in asthmatic subjects is supported by a cross-sectional study of asthmatic schoolchildren reporting increased IL-5 and IL-13 levels in the immune responses to inhaled allergens.²⁰ Moreover, biological antagonists have demonstrated clinical relevance of IL-5 and IL-13 by reducing exacerbations and eosinophilia, and by increasing lung function.¹⁰⁻¹²

Inefficient clearance of bacteria in asthmatic subjects might contribute to an understanding of our earlier observation of an association between neonatal bacterial airway colonization and asthma at age 5 years.³ The current findings suggest an inappropriate immune response to pathogenic bacteria in the airways preceding the development of asthma. This might permit persistent colonization, triggering chronic airway inflammation characterized by the type 2 cytokine-mediated immunopathology of asthma.²¹

Our study was not designed to address the origins of the aberrant immune function associated with development of asthma, which might in part be genetic. Interestingly, a recent line of experimental work in mice has demonstrated that early commensal bacterial exposure decreases asthma presentation through epigenetic imprinting of immune function.^{22,23}

It should be noted that asthma is a highly heterogeneous disease consisting of many yet incompletely understood endotypes.¹ The disease mechanism we propose here, in which an adverse immune reaction to bacterial exposure seems to play a role, is likely important for some but perhaps not all endotypes. Orosomucoid 1-like protein 3 (ORMDL3) is an example of an endotype driven

by a genetic variant with a very high risk of early-onset childhood asthma but not adult asthma.²⁴ Interestingly, the risk for asthma by ORMDL3 variation was recently found to be specifically attributable to prior human rhinovirus-induced respiratory wheezing, demonstrating a novel gene-environment interaction in asthmatic patients.²⁵ Bacteria-driven immunopathology in asthmatic subjects might likewise be limited to a particular childhood endotype with specific genetic predisposition.

Children with asthma by school age exhibited an inappropriate immune response to pathogenic bacteria in infancy characterized by asthma-associated type 2 responses and suppressed antibacterial immunity. This finding is consistent with inefficient bacterial eradication, which might initiate the chronic airway inflammation characteristic of asthma.

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Clinical implications: Abnormal immune responses to pathogenic airway bacteria underlie the development of asthma in childhood. Novel strategies to promote appropriate bacterial immunity in infancy might have implications for disease prevention.

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METHODS

PBMC preparation

Up to 4 mL of peripheral blood from 6-month-old infants was drawn into heparin-coated tubes. The blood was diluted 1:1 in RPMI 1640 medium (Lonza, Basel, Switzerland) and layered into Lymphoprep (AXIS-SHIELD PoC, Oslo, Norway). Cells were centrifuged at 800g for 30 minutes at room temperature for the separation of PBMCs. PBMCs were collected from the Lymphoprep/plasma interphase, washed twice in medium supplemented with 1% FBS, and frozen in medium supplemented with 20% FBS and 10% dimethyl sulfoxide. PBMCs were stored at -140°C until analysis.

Preparation of bacteria

Nontypeable *H influenzae* (KAK509), *M catarrhalis* F48 (KAK508), and *S pneumoniae* serotype 6A/2 (KAK511) reference strains were kindly provided by Karen Krogfelt, Statens Serum Institut, Copenhagen, Denmark. *H influenzae* and *M catarrhalis* were grown on chocolate agar plates (Statens Serum Institut). *S pneumoniae* was grown on 10% blood agar plates (Statens Serum Institut). All strains were grown under 37°C microaerobic conditions (5% CO_2) for 24 hours after plating. Afterward, each strain was resuspended from plates with uniform growth and washed once in PBS. Bacteria were resuspended in PBS to OD 1 and UV-irradiated for 45 minutes. UV killing was confirmed by using plating. Dry weights of bacterial suspensions were determined after freeze-drying by subtracting the weight of PBS alone. Bacterial suspensions were frozen and stored at -80°C .

PBMC bacterial stimulation

PBMC samples were chosen randomly for stimulation and quickly thawed in a 37°C water bath until just a small piece of ice was left. The PBMCs were gently transferred to 37°C RPMI 1640 completed medium (2 mmol/L L-glutamine [Cambrex, East Rutherford, NJ], 0.1 mol/L HEPES (Lonza), and 100 U/mL penicillin/streptomycin [Lonza]) supplemented with 20% FBS. Cells were counted with NucleoCounter equipment (ChemoMetec, Allerød, Denmark), washed once, and resuspended to 5×10^6 cells/mL in RPMI 1640 medium supplemented with 10% FBS. PBMCs were plated in U-bottom 96-well plates at 5×10^5 cells/well, stimulated in duplicates (singles if insufficient material) with 50 $\mu\text{g/mL}$ bacterial preparations or medium alone (200 μL total volume/well), and incubated for 40 hours (37°C in a 5% CO_2 atmosphere). After incubation, supernatants were harvested and stored at -80°C until analysis. T-cell activation was analyzed in PBMCs (pellet) immediately after incubation using flow cytometry.

T-cell activation analysis

Stimulated PBMC duplicates were pooled and washed once in fluorescence-activated cell sorting (FACS) buffer (1% FBS and 0.1%

Na-azide in PBS). Cells were resuspended in 50 μL of FACS buffer supplemented with 10% heat-inactivated human AB serum and incubated for 5 minutes at 4°C . A pretitrated fluorochrome-conjugated antibody mixture designed to analyze T-cell activation (see Table E2) was added, and cells were incubated for another 30 minutes at 4°C . After incubation, cells were washed once and resuspended in 200 μL of FACS buffer. Cells were analyzed on a BD FACSCanto II Flow Cytometer running FACSdiva 6.0 software (BD Biosciences, San Jose, Calif). The percentage of T cells expressing OX40, CD25, or CD69 was analyzed in a blinded manner by using a predefined gating strategy. T-cell activation was analyzed for both helper and cytotoxic T cells by separating the cells based on their expression of CD4 and CD8, respectively. Cytotoxic T cells were not analyzed for their expression of OX40 because this activation marker is predominantly expressed by helper T cells.

Immune phenotyping

Unstimulated PBMCs (5×10^5) were washed once in FACS buffer. Cells were stained with a pretitrated fluorochrome-conjugated antibody mixture designed to characterize the T-cell compartment (see Table E3) and analyzed as described above. Helper ($\text{CD}3^{+}\text{CD}4^{+}$), cytotoxic ($\text{CD}3^{+}\text{CD}8^{+}$), regulatory ($\text{CD}3^{+}\text{CD}4^{+}\text{CD}127^{-}\text{CD}25^{+}$), $\gamma\delta$ ($\text{CD}3^{+}\text{TCR}\gamma\delta^{+}$), and invariant NK ($\text{CD}3^{+}\text{TCRV}\alpha 24\text{-J}\alpha 18^{+}$) T cells were analyzed in a blinded manner by using a predefined gating strategy. Phenotypic T-cell subsets were expressed as percentages of the full $\text{CD}3^{+}$ T-cell population.

PCA

Let X be a matrix of 993 (331×3) samples and 7 variables ($X = [X_{\text{H1}}; X_{\text{M1}}; X_{\text{S1}}]$). Each of the 331 children is represented by 3 samples, one for each bacterial stimulation. The matrix X is decomposed into a set of PCs (k) that optimally, in a least-squares sense, describe the data (see equation 1). Be aware that this is done without any knowledge concerning the outcome.

$$X = TP' + E \quad (\text{equation 1})$$

P ($7 \times k$) is the loading matrix reflecting common cytokine patterns across all 3 bacterial stimulations, and T ($993 \times k$) is the score matrix reflecting how much a given sample has of the corresponding pattern. For example, let us assume 2 components are extracted reflecting 2 different immune patterns. Then each child will have a set of 6 score values (2×3), one for each combination of pattern and bacteria stimulation. Examination of the scores (T) reveals which sources of information are responsible for the dominating part of the data variation. The extracted score values can be used as compressed measures of the total data information (12 variables) in a regression context versus, for example, asthma at 7 years.

TABLE E1. Cohort baseline

		Full COPSAC ₂₀₀₀	Dropout	Study cohort	P value
Child					
Male sex	Mean	49%	44%	51%	.261*
	No.	411	80	331	
Gestational age	Mean	39.90 wk	39.71 wk	39.95 wk	.233†
	SD	1.57 wk	1.66 wk	1.54 wk	
	No.	411	80	331	
	Mean	3.52 kg	3.49 kg	3.52 kg	
Birth weight	SD	0.52 kg	0.54 kg	0.52 kg	.604†
	No.	411	80	331	
Birth length	Mean	52.30 cm	52.09 cm	52.35 cm	.362†
	SD	2.31 cm	2.37 cm	2.20 cm	
	No.	411	80	331	
	Mean	21%	21%	21%	
Cesarean section	No.	411	80	331	.889*
	Mean	88%	89%	88%	
Apgar score >9	No.	411	80	331	.894*
	Parents				
Mother's asthma history	Mean	100%	100%	100%	NA
	No.	411	80	331	
Mother's rhinitis history	Mean	76%	78%	75%	.508*
	No.	410	79	331	
Father's asthma history	Mean	17%	19%	17%	.567*
	No.	399	78	321	
Father's rhinitis history	Mean	33%	32%	33%	.770*
	No.	397	76	321	
Mother's age at birth	Mean	30.00 y	29.40 y	30.15 y	.184†
	SD	4.55 y	4.69 y	4.50 y	
	No.	411	80	331	
	Mean	31.99 y	31.39 y	32.14 y	
Father's age at birth	SD	5.17 y	5.44 y	5.10 y	.253†
	No.	401	79	322	
Mother's occupation (nonprofessional)	Mean	33%	34%	33%	.919*
	No.	376	56	320	
Mother's occupation (professional)	Mean	46%	48%	46%	
	No.	376	56	320	
Mother's occupation (student)	Mean	11%	9%	11%	
	No.	376	56	320	
Mother's occupation (unemployed)	Mean	10%	9%	10%	
	No.	376	56	320	
Mother's highest education (college)	Mean	60%	48%	62%	.062*
	No.	376	56	320	
Mother's highest education (medium)	Mean	27%	39%	24%	
	No.	376	56	320	
Mother's highest education (university)	Mean	14%	13%	14%	
	No.	376	56	320	
Household income	Mean	494,029 DKK	458,350 DKK	500,615 DKK	.146†
	SD	206,581 DKK	210,132 DKK	205,569 DKK	
	No.	385	60	325	
Perinatal environment					
Mother smoking, 3rd trimester	Mean	15%	18%	15%	.548*
	No.	411	80	331	
Antibiotics, 3rd trimester	Mean	14%	18%	14%	.371*
	No.	411	80	331	
Furred animals, 3rd trimester	Mean	29%	30%	29%	.903*
	No.	411	80	331	
Furred animals, 1st year of life	Mean	72%	70%	72%	.694*
	No.	411	80	331	
Older siblings	Percent	37%	29%	39%	.099*
	No.	411	80	331	

NA, Not applicable.

*Pearson χ^2 test.

†Student *t* test.

TABLE E2. Antibodies used for flow cytometric analysis of T-cell activation

Antigen	Conjugate	Clone	Manufacturer
CD3	eFlour450	UCHT1	eBioscience, San Diego, Calif
CD4	V500	RPA-T4	BD Biosciences, San Jose, Calif
CD8	Fluorescein isothiocyanate	OKT8	eBioscience
OX40 (CD134)	Phycoerythrin	Ber-ACT35	Beckman Coulter, Brea, Calif
CD25	PC7	B1.49.9	Beckman Coulter
CD69	Allophycocyanin	TP1.55.3	Beckman Coulter

TABLE E3. Antibodies used to characterize the T-cell compartment by using flow cytometry

Antigen	Conjugate	Clone	Manufacturer
CD3	eFlour450	UCHT1	eBioscience, San Diego, Calif
CD4	V500	RPA-T4	BD Biosciences, San Jose, Calif
CD8	Fluorescein isothiocyanate	OKT8	eBioscience
CD25	PC7	B1.49.9	Beckman Coulter, Brea, Calif
CD127	Allophycocyanin-eFlour780	A7R34	eBioscience
TCR $\gamma\delta$	Phycoerythrin	IMMU510	Beckman Coulter
TCRVa24-Ja18	PerCP-eFlour710	6B11	eBioscience