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.)

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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 ($COPSAC_{2000}$) 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 $COPSAC_{2000}$ 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 pneumonia*.

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 $COPSAC_{2000}$ 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

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 $COPSAC_{2000}$ 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 pneumonia*, 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 ($CD3^+CD4^+$), cytotoxic ($CD3^+CD8^+$), regulatory ($CD3^+CD4^+CD127^-CD25^+$), γδ ($CD3^+$ T-cell receptor [TCR]$\gamma\delta^+$), and invariant natural killer (NK; $CD3^+TCRV\alpha24\text{-}J\alpha18^+$) 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 $COPSAC_{2000}$ 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, regulatory, γδ, 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*.