Season of birth shapes neonatal immune function

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Background: Birth season has been reported to be a risk factor for several immune-mediated diseases. We hypothesized that this association is mediated by differential changes in neonatal immune phenotype and function with birth season.
Objective: We sought to investigate the influence of season of birth on cord blood immune cell subsets and inflammatory mediators in neonatal airways.
Methods: Cord blood was phenotyped for 26 different immune cell subsets, and at 1 month of age, 20 cytokines and chemokines were quantified in airway mucosal lining fluid. Multivariate partial least squares discriminant analyses were applied to determine whether certain immune profiles dominate by birth season, and correlations between individual cord blood immune cells and early airway immune mediators were defined.
Results: We found a birth season–related fluctuation in neonatal immune cell subsets and in early-life airway mucosal immune function. The seasonal airway immune pattern was associated with the number of activated and regulatory T cells in cord blood whereas it was independent of concomitant presence of pathogenic airway microbes. Specifically, summer newborns presented with the lowest levels of all cell types and mediators; fall newborns displayed high levels of activated T cells and mucosal IL-12p70, TNF-α, IL-13, IL-10, and IL-2; and winter newborns had the highest levels of innate immune cells, IL-5, type 17–related immune mediators, and activated T cells.
Conclusion: Birth season fluctuations seem to affect neonatal immune development and result in differential potentiation of cord blood immune cells and early airway mucosal immune function. (J Allergy Clin Immunol 2016;137:1238-46.)

Key words: *Birth season, cord blood immune cells, airway mucosa, neonatal immunity*

Several immune-mediated diseases have birth season as a common risk factor,[1-3] although the critical season varies depending on the disease. Autoimmune diseases are generally associated with spring births,[1,4,5] whereas asthma and allergies are more common among subjects born in fall and winter.[2,3] Season-related environmental encounters in perinatal life or absence thereof might be important for later disease development,[6-9] and the timing of these events can be central.[10,11] Because many of these diseases, such as asthma, rheumatoid arthritis, and multiple sclerosis, have an underlying immune-mediated pathology, we hypothesized that birth season might shape neonatal immune phenotype and function. In turn, this could affect the underlying risk of disease.

Neonatal immunology seems to be more strongly associated with birth season than with other independent birth variables, including sex, birth weight, and cesarean delivery,[12-14] when studied by using measures of overall leukocyte counts and production of selected cytokines after *ex vivo* stimulation. Still, it is unclear whether seasonal programming events differentially influence the prevailing immune phenotype at birth, thereby affecting the ability to handle microbial exposure at mucosal surfaces in early life. Combined, these events might influence the susceptibility for later disease development differently.

This study was designed to characterize multiple functional aspects of the immune system in relation to birth season by using statistical pattern recognition tools. We based the analysis on a detailed characterization of the neonatal immune system, including a comprehensive phenotypic analysis of cord blood immune cell subsets, as well as *in situ* production of several cytokine and chemokine mediators in the upper airway mucosa of the neonate. A combined analysis of the 2 immune response types allowed for identification of those cord blood immune cells that associate with the response profile in the upper airways at 1 month of age.

METHODS

Study cohort

The study is nested within the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) 2010 birth cohort, an ongoing unselected clinical prospective birth cohort of 700 children.[15] Informed consent was obtained from both parents before recruitment of the children. Children were enrolled during 2009 and 2010. The study was performed in agreement with the guiding principles of the Declaration of Helsinki and approved by the Ethics Committee for Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (j.nr. 2008-41-2599).

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Abbreviations used
BDCA: Blood dendritic cell antigen
COPSAC: Copenhagen Prospective Studies on Asthma in Childhood
DC: Dendritic cell
GLM: Generalized linear model
iNKT: Invariant natural killer T
LV: Latent variable
NK: Natural killer
pDC: Plasmacytoid dendritic cell
PLSDA: Partial least squares discriminant analysis
SSC: Side scatter
TCR: T-cell receptor
Treg: Regulatory T

Season of birth was defined as spring (March-May), summer (June-August), fall (September-November), and winter (December-February).

Baseline characteristics were recorded by trained physicians during scheduled visits at the COPSAC research clinics at gestational weeks 24 or 36 or 1 week postpartum.

Flow cytometry of cord blood immune phenotypes

By use of a lyse-no-wash procedure, umbilical cord blood was stained with pretitrated antibody mixtures (see Table E1 in this article's Online Repository at www.jacionline.org). Cell subsets were predefined based on specific surface markers as follows: leukocytes ($CD45^+$), granulocytes ($CD45^+$ side scatter $[SSC]^{int/high}$), neutrophils ($CD45^+CD16^+SSC^{int/high}$), eosinophils ($CD45^+CD16^-SSC^{high}$), B cells ($CD45^+CD19^+$), T cells ($CD3^+$), CD4 T cells (αβ T-cell receptor $[TCR]^+CD4^+$), CD8 T cells ($\alpha\beta TCR^+CD8^+$), activated CD4 T cells ($\alpha\beta TCR^+CD4^+CD25^+CD127^{high/+}$), activated CD8 T cells ($\alpha\beta TCR^+CD8^+CD25^+CD127^{high/+}$), regulatory T (Treg) cells ($\alpha\beta TCR^+CD4^+CD25^+CD127^{low/-}$), γδ T cells ($\gamma\delta TCR^+$), invariant natural killer T (iNKT) cells ($CD3^+V\alpha24J\alpha18^+$), classical monocytes ($CD3^-CD19^-CD56^-CD14^{+/high}CD16^-$), intermediate monocytes ($CD3^-CD19^-CD56^-CD14^{+/high}CD16^+$), inflammatory monocytes ($CD3^-CD19^-CD56^-CD14^{int/-}CD16^+$), blood dendritic cell antigen (BDCA)-1 dendritic cells (DCs; $CD3^-CD19^-CD56^-CD14^-CD16^-BDCA\text{-}1^+$), BDCA-3 DCs ($CD3^-CD19^-CD56^-CD14^-CD16^+CD1c^-CD303^-BDCA\text{-}3^{high}$), plasmacytoid dendritic cells (pDCs; $CD3^-CD19^-CD56^-CD14^-BDCA\text{-}2^+$), natural killer (NK) cells ($CD3^-CD56^+$), CD56d NK cells ($CD3^-CD56^{dim}$), and CD56b NK cells ($CD3^-CD56^{bright}$), as well as overall numbers of monocytes and DCs. Adaptive immune cells were defined as the sum of T and B cells, and innate immune cells were defined as the sum of NK cells, granulocytes, monocytes, and DCs. Cell events were gated by using a predefined gating strategy (see Fig E1 in this article's Online Repository at www.jacionline.org).

Airway mucosal cytokines and chemokines

Mucosal lining fluid was sampled from both nostrils onto filter paper (Accuwik Ultra, fibrous hydroxylated polyester sheets, catalog no. SPR0730; Pall Life Sciences, Portsmouth, United Kingdom), and extracted cytokine and chemokine levels were measured in duplicates by using Meso Scale Discovery multiplex assays, as detailed previously.[16,17] Detectable inflammatory mediators comprised CCL2 (monocyte chemoattractant protein 1), CCL4 (macrophage inflammatory protein 1β), CCL11 (eotaxin-1), CCL13 (monocyte chemoattractant protein 4), CCL17 (thymus and activation-regulated chemokine), CCL22 (macrophage-derived chemokine), CCL26 (eotaxin-3), CXCL8 (IL-8), CXCL10 (interferon-inducible protein 10), IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A (IL-17), TGF-β1, and TNF-α.

Statistics

Pearson χ^2 tests or ANOVA were used for simple baseline statistics. Cells were expressed as absolute counts (10^6/mL), and airway mediator concentrations were determined as pg/mL. Undetectable airway mediator levels were set to half of the minimal non-zero registrations. Both cell and airway mediator concentrations were log transformed and scaled to z scores before being subjected to statistical analysis.

Coincident colonization of the upper airways with *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae* results in generally increased levels of immune mediators in the airway mucosa,[17] and the presence of either *H influenzae*, *M catarrhalis*, *S pneumoniae*, picornavirus, influenza virus, and respiratory syncytial virus is associated with winter season in our data set. To remove postnatal and technical effects on inflammatory mediator profiles, z scores were calculated on log-transformed data for each of 4 strata of concurrent colonization of bacteria or virus (yes/no) and batch effects (1/2) before being pooled to a combined data set. A total of 50 children had a concurrent airway infection or unknown colonization status on the day of mucosal lining fluid sampling and were excluded from the statistical analysis.

For each cell population, the variation in birth season was modeled with a Fourier series function and fitted using generalized linear models (GLMs) as follows: $f(x) = a_0 + a\cos\frac{2\pi t}{365} + b\sin\frac{2\pi t}{365}$, where t is defined as days from March 1. For airway inflammatory mediators, some mediators possessed nonnormal residuals (CCL22, CCL26, CXCL8, IFN-γ, and IL-4) after a GLM. Therefore data on individual airway inflammatory mediators were modeled by using robust regression (GLMrob) to minimize type I errors resulting from outliers. Statistical significance was assessed by comparing the fitted model with an intercept model only using the F test for ordinary fittings and the Wald test for robust fittings, respectively.

Partial least squares discriminant analysis (PLSDA)[18] was used to attain discriminatory latent variables (LVs) that should separate newborns by birth season dependent on the systemic variation among cord blood immune cell subsets. The score plot illustrated the division of newborns based on their birth season. The corresponding loading plot showed the cell count–cell count correlation structure. An identical approach was repeated with airway mediators. The number of significant components was evaluated by means of 10-fold cross-validation. Random permutation testing of the overall cross-validated deviance with 10,000 permutations was applied to estimate the statistical inference of each PLSDA model.

The Spearman rho statistic was used to estimate a rank-based measure of correlation between levels of cord blood immune cells and airway immune mediators. The resulting Spearman rho correlation coefficients were plotted by using the corrplot package in R software.

Data were analyzed in R (version 3.0.1), RStudio (version 0.97.320; RStudio, Boston, Mass), and MATLAB R2012b (version 7.9.0.529; Mathworks, Natick, Mass) software with PLS toolbox (version 6.5.1) for model estimation. Visualization permutation testing and Procrustes rotation were done by using in-house algorithms.

Additional specifications on the methodology used are detailed in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

General characteristics of the study group

The children studied are part of the $COPSAC_{2010}$ birth cohort of 700 children.[15] Absolute cell counts were determined in freshly collected cord blood of 84 (12%) infants born between June and February, and upper airway mucosal cytokine and chemokine levels were determined in mucosal lining fluid samples from 620 (89%) 1-month-old children over a period of 2 years (see Fig E2 in this article's Online Repository at www.jacionline.org). General characteristics of infants with cord blood samples and infants with airway mucosal lining fluid samples are shown in Tables E2 and E3 in this article's Online Repository at www.jacionline.org, respectively. Among the 84 children with cord blood samples, history of maternal atopy and birth weight varied with respect to birth season. Likewise, the frequency of mode of delivery, antibiotic use in pregnancy, and household cat during

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