Regulation of T_H17 markers early in life through maternal farm exposure

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Background: Previous studies suggested that maternal farm exposure during pregnancy modulates early immune development toward an allergy-protective status potentially mediated by T_H1 or regulatory T (Treg) cells. However, the underlying mechanisms might involve immune modulation of additional T-cell populations, such as T_H17 cells, influenced by genetic predisposition.

Objective: We examined the role of maternal farm exposure and genetic predisposition on T_H17 cell responses to innate and adaptive immune stimulation in cord blood.

Methods: Eighty-four pregnant mothers were recruited before delivery. Detailed questionnaires (60 nonfarming mother, 22 farming mothers, and 2 exclusions) assessed farming exposures. Cord blood was stimulated with lipid A, peptidoglycan (Ppg), or PHA. T_H17 lineage (retinoic acid receptor–related orphan receptor C *[RORC]*, retinoic acid receptor–related orphan receptor α *[RORA]*, IL-23 receptor *[IL23R]*, *IL17*, *IL17F*, and *IL22*) and Treg cell markers (forkhead box protein 3 *[FOXP3]*, lymphocyte activation gene 3 *[LAG3]*, and glucocorticoid-induced TNF receptor *[GITR]*) were assessed at the mRNA level. T_H17/Treg/T_H1/T_H2 cytokines and 7 single nucleotide polymorphisms within the T_H17 lineage (*RORC*, *IL23R*, and *IL17*) were examined.

Results: T_H17 lineage mRNA markers were expressed at birth at low concentrations independent of maternal farm exposure. A positive correlation between T_H17 lineage markers and *FOXP3* (mRNA) was observed on stimulation (nonfarming mothers: lipid A, Ppg, and PHA; farming mothers: Ppg and PHA), influenced by maternal farming. Specific single nucleotide polymorphisms within the T_H17 lineage genes influenced gene expression of T_H17 and Treg cell markers and cytokine secretion.

Conclusions: Gene expression of T_H17 lineage markers in cord blood was not influenced by maternal farming. Yet T_H17 and Treg cell markers were positively correlated and influenced by maternal farm exposure. Our data suggest that prenatal exposures and genetic predisposition play a role during early T_H17 immune maturation, potentially regulating the development of immune-mediated diseases, such as childhood asthma. (J Allergy Clin Immunol 2014;133:864-71.)

Key words: *Cord blood, cytokines, farming, innate, single nucleotide polymorphism, T_H17 cells, regulatory T cells*

T_H17 cells represent a $CD4^+$ cell subset important in host defense against bacterial infection; however, they also play a role in the pathogenesis of immune-mediated diseases, including atopic diseases (ADs).[1-3] IL-17–producing T-cell numbers were increased in patients with ADs.[3-6] Subjects affected by ADs had allergic hypersensitivity and enhanced serum IgE levels and might have atopic eczema, asthma, and/or hay fever.[7]

ADs summarize complex multifactorial diseases, and the interplay of genetic susceptibility and environmental factors most likely influence their development, potentially through modulation of the immune system. In this context maternal atopy has been identified as a major risk factor for ADs in childhood,[8,9] whereas farm[10,11] or farm milk[12-14] exposure conferred protection. Indeed, the farm effect was stronger when exposure occurred early in life, particularly prenatally.[12,15] Furthermore, maternal farm exposure during pregnancy was associated with increased numbers of regulatory T (Treg) cells and more efficient suppressive activity and decreased T_H2 cytokine secretion after innate exposure in cord blood.[16] In addition to Treg cells, additional T-cell subpopulations might be critical.

T_H17 and Treg cells are reported to be reciprocally regulated.[17,18] However, little is known about T_H17 cells in early life in an allergy-protective environment, such as farming exposure. Although no significant differences were observed in IL-17 secretion in cord blood depending on maternal farming exposure, this might be explained by low protein expression early in life.[16] A more detailed characterization of T_H17 cells at this early stage of immune maturation might help elucidate their role in early-life immune maturation and potential development of ADs.

In this study we hypothesized that cord blood mononuclear cells (CBMCs) of offspring of farming mothers can show decreased T_H17 cell numbers potentially regulated by genetic predisposition, with subsequent effects on early immune

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Abbreviations used
AD: Atopic disease
CBMC: Cord blood mononuclear cell
CT: Cycle threshold
FOXP3: Forkhead box protein 3
GITR: Glucocorticoid-induced TNF receptor
IL-23R: IL-23 receptor
LAG3: Lymphocyte activation gene 3
LpA: Lipid A
Ppg: Peptidoglycan
RA: Retinoic acid
RORA: Retinoic acid receptor–related orphan receptor α
RORC: Retinoic acid receptor–related orphan receptor C
SNP: Single nucleotide polymorphism
Treg: Regulatory T

regulation. Therefore distinct protein and mRNA markers of different T-cell subsets, including T_H17, Treg, T_H1, and T_H2 cells, were analyzed in cord blood on innate and mitogen stimulation. Single nucleotide polymorphisms (SNPs) located within the T_H17 lineage genes were assessed.

METHODS

Population characteristics

Cord blood (n = 84) was sampled from a birth cohort study performed in the rural area of Munich, Germany (PAULCHEN[16]). Enrollment occurred from July 2005 to September 2007. Pregnant mothers were approached for consent before delivery and completed a detailed questionnaire that assessed maternal and infant data. Cord blood was obtained from healthy neonates born without complications, excluding children with signs of infection, severe chronic maternal disease, and/or maternal intake of medication during pregnancy. Eighty-two samples were included (60 nonfarming mothers, 22 farming mothers, and 2 exclusions). Maternal farm exposure was defined as the mother living and regularly working on a farm in the last 5 years and during pregnancy; their children were defined as farming children accordingly. Specific exposures to stables/barns, animal species, and milk intake were documented during pregnancy. For retinoic acid (RA) experiments, CBMCs of 7 healthy control children were recruited randomly at the Munich University Maternity Hospital (Munich, Germany). Approval was obtained from the local review board (Bavarian Ethical Board, Germany).

Isolation, lymphocyte proliferation, and cytokine secretion of CBMCs

CBMCs were isolated within 24 hours by using density gradient centrifugation with Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden), as previously described,[19] and stimulated with lipid A (LpA; 0.1 μg/mL), peptidoglycan (Ppg; 10 μg/mL), or PHA (5 μg/mL) for 3 days in comparison with unstimulated cells.[19] Cytokine concentrations were measured in supernatants by using the Human Cytokine Multiplex Assay Kit, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, Calif), applying Luminex technology (Luminex, Austin, Tex). The lower limit of detection of the assay was 1.1 pg/mL for IL-2, 1.8 pg/mL for IL-5, 0.5 pg/mL for IL-6, 3.0 pg/mL for TNF-α, 0.9 pg/mL for IL-10, 2.1 pg/mL for IL-13, 4.2 pg/mL for IL-15, 0.2 pg/mL for IL-17, 1.3 pg/mL for IFN-γ, and 1.0 pg/mL for GM-CSF. Endotoxin concentrations in Ppg and PHA, as measured by using the Limulus assay, were low (<0.01 EU/mL = 0.002 ng/mL) and did not significantly change cytokine secretion.

RA stimulation of CBMCs

T-cell responses to RA were analyzed in CBMCs. Both unstimulated and PHA-stimulated (5 μg/mL) CBMCs were incubated with or without RA. Three RA doses were used: 2.5, 50, and 100 nmol/L. Cells were harvested after 48 hours for mRNA expression analysis of forkhead box protein 3 *(FOXP3)*, retinoic acid receptor–related orphan receptor C *(RORC)*, and IL-23 receptor *(IL23R)*.

Quantitative real-time RT-PCR

Total RNA was isolated with TRI Reagent (Invitrogen, Carlsbad, Calif), and reverse transcription of 1 μg of RNA was performed, according to the manufacturer's instructions (Qiagen, Hilden, Germany). mRNA-specific oligonucleotide primers (FW/RE) of T_H17 lineage–related markers, including the transcription factors *RORC* and retinoic acid receptor–related orphan receptor α *(RORA)*; the cytokines *IL17*, *IL17F*, and *IL22*; and the transmembrane receptor *IL23R* and Treg cell markers *FOXP3*, glucocorticoid-induced TNF receptor *(GITR)*, and lymphocyte activation gene 3 *(LAG3)*, were designed with Vector NTI Advance10 (Invitrogen, Carlsbad, Calif; see Table E1 in this article's Online Repository at www.jacionline.org). Direct detection of the PCR product (iCycler, Hercules, Calif) was monitored by measuring the increase in fluorescence caused by binding of SYBR Green (Applied Biosystems, Foster City, Calif) to double-stranded DNA. For analyses, the determined cycle threshold (CT) was set in relation to the amplification plot of *18S* rRNA. The CT is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value, which was set to the log-linear range of the amplification curve. The difference in CT values of 2 genes was used to calculate ΔCT. A higher ΔCT resembles lower mRNA expression.[20,21]

Polymorphism selection and genotyping

Genotyping data for SNPs in *IL17*, *IL23R*, and *RORC* (Entrez Gene IDs 3605, 149233, and 6097, respectively), including approximately 5-kb borders upstream and downstream of each gene region, were extracted from the MAGICS/ISAAC discovery data set.[22] By using PLINK software package version 1.07,[23] SNPs associated with asthma in the MAGICS/ISAAC discovery data set were identified as reported elsewhere[22] and genotyped in the PAULCHEN study population for functional assessment. Additionally, rs2275913 was included in this study because of its potential functional relevance in IL-17 regulation and asthma (Table I).[24,25] Genotyping was performed by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom, San Diego, Calif) at the Helmholtz Centre Munich (Neuherberg, Germany), as previously described.[26] Further technical information on assay design is available from the authors on request. Genotyping call rates were 96.7% or greater, no deviations from Hardy-Weinberg equilibrium were detectable, and the level of significance was set to a *P* value of .05 or less.

Statistical analysis

All statistical analyses were performed with the SAS statistical software package (version 9.2; SAS Institute, Cary, NC), with statistical significance set at a *P* value of less than .05.

Data from gene expression and cytokine secretion at the protein level were analyzed by using nonparametric statistical methods, taking censored observations into account because not all variables could be transformed into normality and data contained nondetectable observations.[27] Therefore summary statistics were conducted by using the Kaplan-Meier method.[28] Testing on group differences without adjusting for covariates was performed with the generalized Wilcoxon test.[29] For comparison of paired censored observations, the paired Prentice-Wilcoxon test was performed.[30] The Tobit model[31] was applied to the ranks of the original data to adjust for covariates. Covariates were exposure to stables, exposure to barns, maternal education, and smoking status and included in the models. Application of these methods to environmental settings with censored data is recommended by Nondetects and Data Analysis.[32] Maternal atopy was not a confounder and thus not included as a covariate. Correlations between Treg cell and T_H17 mRNA markers were assessed by using the Spearman rank correlation coefficient. In association with T_H17 lineage SNPs, T_H17 and Treg cell markers were