Impairment of T-regulatory cells in cord blood of atopic mothers

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Background: Maternal atopy is a strong predictor for the development of childhood allergic diseases. The underlying mechanisms are ill defined, yet regulatory T (Treg) and T_H17 cells may play a key role potentially shaping the early immune system toward a proallergic or antiallergic immune regulation.

Objective: We examined T_H1/T_H2, Treg, and T_H17 cell responses to innate (lipid A/peptidoglycan) and mitogen/adaptive (phytohemagglutinin/*Dermatophagoides pteronyssinus* 1) immune stimulation in cord blood from offspring of atopic/nonatopic mothers.

Methods: Cord blood mononuclear cells from 161 healthy neonates (59% nonatopic, 41% atopic mothers) were investigated regarding Treg and T_H17 cells (mRNA/surface markers), suppressive function, and proliferation/cytokine secretion.

Results: Cord blood from offspring of atopic mothers showed fewer innate-induced Treg cells ($CD4^+CD25^+$high), lower mRNA expression of associated markers (glucocorticoid-induced tumor necrosis factor receptor–related protein/lymphocyte activation gene 3; $P < .05$), and a trend toward lower Forkhead box transcription factor 3 (Foxp3) expression. Treg cell function was impaired in mitogen-induced suppression of T effector cells in cord blood of offspring from atopic mothers ($P = .03$). Furthermore, IL-10 and IFN-γ secretion were decreased in innate-stimulated cord blood of offspring from atopic mothers ($P = .04/.05$). Innate-induced IL-17 was independent of maternal atopy and highly correlated with IL-13 secretion.

Conclusion: In offspring of atopic mothers, Treg cell numbers, expression, and function were impaired at birth. T_H17 cells were correlated with T_H2 cells, independently of maternal atopy. (J Allergy Clin Immunol 2008;121:1491-9.)

Key words: *Adaptive, cord blood, cytokines, innate, IL, regulatory T cells, T_H17 cells, Toll-like receptor*

Abbreviations used

CBMC:	Cord blood mononuclear cell
cpm:	Counts per minute
CT:	Threshold cycle
CTLA4:	Cytotoxic T-lymphocyte–associated protein 4
D+L:	*Dermatophagoides pteronyssinus* 1 and lipid A
FITC:	Fluorescein isothiocyanate
GITR:	Glucocorticoid-induced TNFR-related protein
IQR:	Interquartile range
LAG3:	Lymphocyte activation gene 3
LpA:	Lipid A
OR:	Odds ratio
SI:	Stimulation index
Treg:	Regulatory T
TLR:	Toll-like receptor

Maternal atopy is a critical risk factor for the development of childhood allergic diseases[1] potentially by modulating the child's immune system. However, underlying immunological mechanisms are not well determined. Epidemiologic studies suggest that immune modulation and potential protection against allergies may occur early in life.[2] Maternal atopy may be one important factor interacting with environmental stimuli influencing a child's immune system at an early time point, perhaps even *in utero*.[3]

One potential explanation for the allergy development in offspring of atopic mothers is that immunological mechanisms regulating T_H1/T_H2 pathways may be insufficiently balanced. There is compelling evidence for additional regulatory mechanisms besides the proallergic T_H2 and allergy-protective T_H1 pattern, namely through regulatory T (Treg) and T_H17 cells. A change in amount or function of Treg cells, which play a crucial role in regulating of T_H1/T_H2 effectors and in suppressing default immune pathways, may also contribute to the increase of T_H2/T_H1-mediated immune diseases over the last decades.[4] Treg cells were suggested to be less mature in early than adult life, yet functional differences depending on maternal atopy may influence a child's immune development.[5,6] T_H17 cells, also potentially involved in allergies,[7,8] are a recently identified T-cell population that regulate immune responses in interaction with Treg cells distinct from T_H1/T_H2 cells.[7,8] To our knowledge, the role of T_H17 cells early in life has not been studied in the context of maternal atopy.

Regarding early immune modulation, immediate innate (in addition to adaptive) stimulation may be critical. It has repeatedly been shown that children growing up in environments rich in microbial stimuli, particularly in the first year of life, are at lower risk of allergies.[2,9] Toll-like receptor (TLR)–2 and TLR4, 2 key receptors (for peptidoglycan and lipid A) of innate immune activation, have been shown to be differentially regulated in mice and

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children early in life[10,11] and were increased in children exposed to high amounts of innate microbial stimuli.[12,13]

Here, we aimed to examine cord blood for differences in T-cell subpopulations under different conditions of TLR2, TLR4, and mitogen/adaptive stimulation. We specifically examined whether a maternal atopic background may set the stage for distinct immune modulation in the offspring. We assessed the repertoire of immune responses of T_H1/T_H2 and Treg cells, primarily characterized as $CD4^+CD25^+$high ($Foxp3^+$) T cells, and T_H17 cells after innate and adaptive/mitogen stimulation at birth. We hypothesized that cord blood mononuclear cells (CBMCs) of offspring from atopic mothers may elicit impaired immune responses after stimulation of both innate and adaptive/mitogen activation, potentially resulting in a modulation of early immune responses.

METHODS

For details please see the Methods section in this article's Online Repository at www.jacionline.org.

Study population

Cord blood (n = 161) was sampled from a birth cohort study (Pediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies) in Munich, Germany. Enrollment occurred from January 2005 through February 2007. Pregnant mothers were approached before delivery for consent and completed a detailed questionnaire that assessed maternal and infant data. Cord blood from healthy neonates born without complications excluding children with signs of infection was obtained from the umbilical vein after delivery. We determined maternal and paternal atopy as the presence or absence of an allergic illness as assessed by a doctor's diagnosis of asthma and/or eczema and/or hay fever. In addition, we measured total and specific IgE (RAST). A positive specific IgE was defined as 1 or more positive reactions $\geq$0.7 IU/mL to a panel of 20 common allergens. Potential covariates, including smoking, sex, birth characteristics, race/ethnicity, siblings, education, previous cesarean section, and miscarriage were determined by questionnaire and confirmed through medical records. Birth weight, length, head circumference, and 5-minute Apgar score were recorded at delivery. Approval was obtained from the local review board (Bavarian Ethical Board), Germany.

Lymphocyte proliferation, cytokine secretion of CBMCs

Cord blood mononuclear cells, isolated within 24 hours, were stimulated with lipid A (LpA 0.1 μg/mL), peptidoglycan (10 μg/mL), phytohemagglutinin (5 μg/mL), and *Dermatophagoides pteronyssinus* 1 (30 μg/mL) or a combination of *D pteronyssinus* 1 and LpA (D+L) for 3 days and compared with unstimulated cells.[11,14,15] Significant changes through endotoxin were excluded. After incubation with ^{3}H-Thymidine for additional 8 hours, cells were analyzed for lymphocyte proliferation, assessed by counts per minute (cpm), and quantified by stimulation index (SI), representing the ratio of mean cpm of stimulated/unstimulated replicates. Cytokine concentrations were measured in supernatants by using the Human Cytokine-Multiplex-Assay-Kit according to the manufacturer's instructions (Bio-Rad, Munich, Germany) by LUMINEX technology.

Real-time quantitative RT-PCR

Total RNA, isolated with TRI Reagent (Sigma, Taufkirchen, Germany) was processed with reverse transcriptase (Invitrogen, Karlsruhe, Germany). mRNA-specific oligonucleotide primer pairs were designed with Vector NTI-Advance10 (Invitrogen) (see the Methods section in the Online Repository at www.jacionline.org). Direct detection of the PCR product was monitored by measuring the increase in fluorescence caused by binding of SYBR-Green (Applied Biosystems, Darmstadt, Germany) to double-stranded DNA (dsDNA). The determined threshold cycle (CT) was set relative to the amplification plot of 18S ribosomal RNA (18SrRNA). The CT is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold set to the log-linear range of the amplification curve. The difference in CT values relative to the housekeeping gene was used to calculate the fold difference. The relative quantitative results were used to determine changes of stimulated/unstimulated samples.[16]

Flow cytometry, functional analysis of Treg/T_H17 cells

Cells were analyzed by using 3-color flow cytometry (FACScan; Becton-Dickinson, Heidelberg, Germany). For surface staining, 2 μL antihuman CD4–fluorescein isothiocyanate (FITC), CD25-R-phycoerythrin-Cyanin 5 (RPE-Cy5), 1 μL IgG_1-FITC (Dako Cytomation, Glostrup, Denmark), and 0.5 μL IgG_{2a}-RPE-Cy5 (BD Biosciences, Heidelberg, Germany; Pharmingen, Hamburg, Germany) were used. For intracellular Foxp3/IL-17 staining, 8 μL antihuman CD4-FITC and 4 μL antihuman CD25-RPE-Cy5 antibodies (1×10^6/100 mL) were used, cell permeabilization was performed, and Foxp3-phycoerythrin/antihuman IL-17 antibody/corresponding isotype control was added. Data were analyzed with CellQuest software (Becton-Dickinson), and postacquisition analysis was performed with WinMDI 2.8 software (Becton-Dickinson). Regarding functional Treg cell studies, $CD3^-$ cells were isolated (CD3 isolation kit; Miltenyi Biotec, Köln, Germany) and irradiated. $CD4^+CD25^-/CD4^+CD25^+$ T cells were isolated (2-step procedure) by using depletion of non-$CD4^+$ cells, followed by positive selection of $CD4^+CD25^+$ T cells (Miltenyi Biotec). $CD4^+CD25^-$ T cells (2×10^4/well), labeled with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen), were incubated for 3 days with irradiated $CD3^-$ cells (4×10^4/well) in coculture with/without $CD4^+CD25^+$ T cells (2×10^4/well) before/after stimulation with 0.8 μg/mL phytohemagglutinin. Division and proliferation of $CD4^+CD25^-$ T cells were assessed; cytokine concentrations were measured in supernatants by using the Human Cytokine-Multiplex-Assay-Kit.

Statistical analysis

Data were generally not normally distributed and could regularly not be transformed to normality. Nondetectable cytokine concentrations were assigned to 0.01 pg/mL for inclusion in the analysis. Nonparametric tests (Mann-Whitney/Wilcoxon) were used to compare the median of gene expression, proliferation, and cytokine concentrations; parametric tests were used for normally distributed data (surface expression). Data were reported as either means ± SEMs or medians ± interquartile ranges (IQRs) depending on the distribution. The Spearman correlation was used to assess associations between gene expression and cytokine secretion. χ^2 Tests were used to evaluate categorical predictor variables. Linear, logistic regression analysis was calculated and included potential confounders such as paternal atopy/birth characteristics. The odds ratio (OR) was reported depending on regression analysis. Statistical significance was defined by $P < .05$. Data analysis was performed with SAS 9.1 (SAS, Munich, Germany) and SigmaStat 3.5 software (SigmaStat, Erkrath, Germany).

RESULTS

Population characteristics

Of all healthy mothers (n = 161), complete epidemiologic data and sufficient cord blood for detailed immunological analysis were available from 118 mothers/neonates (73%) complying with the inclusion criteria (Table I; Methods in the Online Repository). Of 118 mothers, 48 mothers were affected by asthma and/or allergic rhinitis and/or atopic eczema. Eighty-five percent of these mothers also had a positive RAST test. The reference group did not have any atopic disease and had sensitization levels comparable to previous epidemiologic studies among nondiseased subjects[17] ($P < .001$). The 2 groups of mothers with and without atopy differed further significantly regarding neonatal birth characteristics ($P \leq .02$)