Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells

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Background: Cross-sectional studies suggest that maternal exposure to farming decreases the risk of allergic diseases in offspring. The potential underlying immunologic mechanisms are not understood.
Objective: We sought to assess whether maternal farm exposure activates regulatory T (Treg) cells in cord blood, exerting T_H2-suppressive effects after microbial stimulation.
Methods: Eighty-four pregnant mothers were recruited before delivery. Detailed questionnaires (60 nonfarming and 22 farming mothers with 2 exclusions) assessed the farming exposures. Cord blood was stimulated with the microbial stimulus peptidoglycan (Ppg), the mitogen PHA, house dust mite extracts (Der p 1), and combinations. Treg cells ($CD4^+CD25^{high}$ cells; intracellular forkhead/winged-helix family transcriptional repressor p3 [FOXP3] expression, FOXP3 levels, lymphocyte activation gene 3 mRNA expression, functional studies, and DNA methylation of the FOXP3 locus), proliferation, and $T_H2/T_H1/T_H17$ cytokines were examined.
Results: Cord blood Treg cell counts (both unstimulated and PHA stimulated) were increased with maternal farming exposures and associated with higher FOXP3 (Der p 1 + Ppg stimulation) and trendwise higher lymphocyte activation gene 3 (Ppg) expression. Furthermore, Treg cell function was more efficient with farming exposure (effector cell suppression, $P = .004$). In parallel, T_H2 cytokine (IL-5) levels were decreased and associated with decreased lymphoproliferation and increased IL-6 levels (Ppg stimulation, Der p 1 + Ppg stimulation, or both; $P < .05$). Maternal exposure to increasing numbers of farm animals and stables was discovered to exert distinct effects on Treg cells, T_H1/T_H2 cells, or both. Additionally, *FOXP3* demethylation in offspring of mothers with farm milk exposure was increased ($P = .02$).
Conclusions: Farm exposures during pregnancy increase the number and function of cord blood Treg cells associated with lower T_H2 cytokine secretion and lymphocyte proliferation on innate exposure. One fascinating speculation is that maternal farm exposure might reflect a natural model of immunotherapy, potentially including a selection of innate stimuli in addition to allergen, shaping a child's immune system at an early stage. (J Allergy Clin Immunol 2009;123:774-82.)

Key words: *Cord blood, cytokines, farming, innate, interleukin, methylation, microbial, T regulatory cells, Toll-like receptor*

One promising natural model of allergy protection has been demonstrated by farm exposure. Children growing up on a farm were shown to have a decreased risk of allergic diseases in later childhood and adulthood.[1] Several cross-sectional studies have reproduced the findings that exposure to stables and barns and consumption of farm milk protects children against the development of atopic diseases (eg, the ALEX and PARSIFAL studies).[2] Furthermore, exposure to an increasing number of animal species was associated with innate Toll-like receptor (TLR) gene expression.[3] In this context maternal farm exposure in pregnancy seems to be critical for the effects seen in these mothers' offspring.[3] Clearly, the influence of farming on early immune development seems to play an important role, promoting the idea of examining infant immune responses at the earliest possible time, namely in cord blood.

Regarding immune modulation in the offspring, one concept of allergy protection is that farm exposure reflects microbial exposure, inducing innate immune mechanism contributing to a less allergic anti-T_H2 immune phenotype. This process likely involves upregulation of different innate receptors (eg, TLR).[4] For example, upregulation of TLR2 expression has been shown in farm children at school age 4 years, and TLR2/TLR4 upregulation was strongly determined by maternal exposure to stables during pregnancy.[3] These human data were supported by murine studies demonstrating decreased allergic responses after TLR2/TLR4 stimulation.[5] However, the specific immunologic mechanisms are not well determined.

Regulatory T (Treg) cells, which play an important role in the balance and maturation of T-cell polarization, are a promising candidate contributing to this effect early in life. Neonatal Treg cells with a naive phenotype have been described to exert a potential immunoregulatory role in intrauterine life.[6,7] To date, Treg cells are best characterized by forkhead/winged-helix family transcriptional repressor p3 (FOXP3) expression, the transcription of which is fundamental for both differentiation and function,[8] complemented by additional markers, such as CD25,

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Abbreviations used
CBMC: Cord blood mononuclear cell
CT: Threshold cycle
FITC: Fluorescein isothiocyanate
FOXP3: Forkhead/winged-helix family transcriptional repressor p3
GITR: Glucocorticoid-induced TNF receptor
LAG3: Lymphocyte activation gene 3
Ppg: Peptidoglycan
TLR: Toll-like receptor
Treg: Regulatory T
TSDR: Treg cell–specific demethylated region

glucocorticoid-induced TNF receptor (GITR), lymphocyte activation gene 3 (LAG3), or low CD127 expression.[9-11] A default regulation or reduced number of $CD4^+CD25^+$ Treg cells might contribute to the development or progression of allergy.[12-14] Indeed, successful treatment of allergy with allergen-specific immunotherapy might depend on induction of Treg cells.[6,15]

The aim of this study was to investigate the immunologic mechanisms contributing to this natural model of allergy protection, namely farm exposure during pregnancy. Specifically, we aimed to examine which exposure might influence neonatal immune development. Next we assessed whether offspring of mothers exposed to farming during pregnancy have quantitatively and qualitatively different Treg cells compared with offspring of nonfarming mothers. We further investigated whether this change would result in different T_H1/T_H2 effector cell responses and lymphocyte proliferation. These experiments were performed with different *in vitro* stimulations, such as microbial TLR ligand and allergen exposure.

METHODS

Study population

We recruited pregnant mothers in an obstetric clinic in rural southern Germany in the frame of a birth cohort study (PAULCHEN). The population is representative of the subjects involved in previous farm studies.[1,3] Study enrollment through trained midwives occurred from July 2005 to September 2007 in the last trimester of pregnancy. Inclusion criteria comprised healthy neonates and mothers with uncomplicated pregnancies. Exclusion criteria included preterm deliveries, perinatal infections, maternal use of antibiotics in the last trimester, and chronic diseases. From 84 mothers enrolled in the study, 82 (97%) cord blood samples were included in the study. Two subjects were excluded because of perinatal infections. Mothers completed a detailed questionnaire regarding rural lifestyle, including detailed farming exposures.[3] Maternal farm exposure was defined as the mother living and regularly working on a farm in the last 1 to 5 years and during pregnancy. Nonfarming mothers lived in the same rural area but not on a farm. Specific exposure to stables/barns and animal species and milk intake were documented during pregnancy. Potential covariates, including delivery mode, sex, birth characteristics, siblings, education, maternal atopy, smoking, and miscarriage, were determined by using a questionnaire. Informed consent was obtained from the mothers for participation in the study, including cord blood collection. Approval was obtained from the local human research committee of the Bavarian Ethical Board, LMU Munich, Germany.

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

Lymphocyte proliferation and cytokine secretion

Cord blood mononuclear cells (CBMCs) freshly isolated within 24 hours were stimulated with peptidoglycan (Ppg; 10 μg/mL), PHA (5 μg/mL), and *Dermatophagoides pteronyssinus* (Der p 1; 30 μg/mL) or a combination of Der p 1 and Ppg (Der p 1 + Ppg) for 3 days and compared with unstimulated cells.[16,17] Dose-response and time curves were assessed in optimization experiments. Significant changes through endotoxin were excluded by functional assays (see the Methods section in this article's Online Repository), and apoptosis was not significantly different in farm-exposed/non–farm-exposed neonates in preliminary experiments. After incubation with tritiated thymidine for 8 hours, cells were analyzed for lymphocyte proliferation, assessed as counts per minute, and quantified based on stimulation index, representing the ratio of mean counts per minute 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), with LUMINEX technology.

Flow cytometry and functional analysis of Treg cells

Cells were analyzed by using 3-color flow cytometry (FACScan; BD Biosciences, Heidelberg, Germany). For surface staining, 2 μL of anti-human CD4–fluorescein isothiocyanate (FITC), 1 microl of CD25–RPE-Cy5, 1 μL of IgG1-FITC (Dako Cytomation, Glostrup, Denmark), and 0.5 μL of IgG2a RPE-Cy5 (BD Biosciences) were added. For intracellular FOXP3 staining, 8 μL of anti-human CD4-FITC and 4 μL of anti-human CD25–RPE-Cy5 antibodies (1×10^6/100 mL) were used, cell permeabilization was performed, and FOXP3-PE/corresponding isotype control antibodies were added. Data were analyzed with CellQuest software (BD Biosciences), and postacquisition analysis was performed with WinMDI 2.8 software (Becton Dickinson, Mountain View, Calif; see Fig E1 in this article's Online Repository at www.jacionline.org). Regarding functional Treg cell studies, $CD3^-$ cells were isolated (CD3 isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany) and irradiated. $CD4^+CD25^-/CD4^+CD25+$ T cells were isolated (as a 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 (Invitrogen, Karlsrhue, Germany) were incubated for 3 days with irradiated $CD3^-$ cells in coculture with or without $CD4^+CD25^+$ T cells before or after stimulation with 0.8 μg/mL PHA. Division and proliferation of $CD4^+CD25^-$ T cells were assessed by means of flow cytometry and tritiated thymidine incorporation, respectively; control experiments showed no significant division/proliferation of $CD4^+CD25^+$ cells; and cytokine concentrations were measured in supernatants by using the Human Cytokine-Multiplex-Assay-Kit (Bio-Rad).

Real-time quantitative RT-PCR

Total RNA isolated with TRI-Reagent was processed with reverse transcriptase (Invitrogen). mRNA-specific oligonucleotide primer pairs were designed (Vector NTI-Advance10). Direct detection of the PCR product was monitored by measuring the increase in fluorescence caused by binding of SYBR Green to dsDNA. The determined threshold cycle (CT) was set relative to the amplification plot of 18SrRNA. CT describes the number of PCR cycles required for the fluorescence signal to exceed the detection threshold, which was set to the log-linear range of the amplification curve. The difference in CT values relative to 18S was used to calculate the fold difference. The formula $2^{-\Delta\Delta CT}$ was applied. Data compare the relative increases compared with values in unstimulated samples.[18]

DNA bisulfite conversion and *FOXP3* methylation–specific real-time-PCR

Genomic DNA was isolated by using the Dneasy blood kit (Qiagen, Hilden, Germany). Bisulfite treatment of genomic DNA was performed.[19] Real-time PCR for measurement of the *FOXP3* Treg cell–specific demethylated region (TSDR)[20] was performed with a 20-μL specimen by using the Roche LightCycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany) containing 15 pmol of methylation-specific or non–methylation-specific forward and reverse primers for TSDR, 5 pmol of hydrolysis probe, 200 ng of lambda-DNA (New England Biolabs, Frankfurt, Germany), and 30 ng of bisulfite-treated genomic DNA template or respective amount of