

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 µL 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 × 10⁶/100 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 × 10⁴/well) labeled with 5 µmol/L Carboxyfluorescein succinimidyl ester (Invitrogen, Karlsruhe, 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 18S rRNA. 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^{-ΔΔCT} 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

TABLE I. Characteristics of the study population

Parameters	Nonfarming CBMCs, (n = 60)	Farming CBMCs (n = 22)	P value*
Maternal age (y), median ($\pm$ IQR)	31.0 (27.0/34.5)	30.5 (24.0/34.0)	.33†
Maternal smoking, no. %			
No	29 (48.3)	18 (81.8)	
Yes, during pregnancy	6 (10.0)	0 (0)	
Yes, until pregnancy	8 (13.3)	2 (9.1)	.04
Yes, only before pregnancy	17 (28.3)	2 (9.1)	
Maternal education, no. (%)			
Elementary school	15 (25.0)	8 (36.4)	
High school	22 (36.7)	12 (54.5)	.06
Grammar school	12 (20.0)	0 (0.0)	
University	11 (18.3)	2 (9.1)	
Maternal atopic diseases, no. %			
Total	19 (31.7)	5 (22.7)	.43
Asthma	3 (5.0)	2 (9.1)	.61
Hay fever	14 (23.3)	2 (9.1)	.21
Atopic eczema	4 (6.7)	1 (4.5)	1.0
Maternal serum total IgE (IU/mL), median ($\pm$ IQR)	34.3 (12.9/121.9)	28.3 (9.7/62.0)	.40†
Vaginal delivery, no. (%)	52 (86.7)	17 (77.3)	.32
Neonatal sex: male, no. (%)	32 (53.3)	10 (45.4)	.53
Gestational age (wk), median ($\pm$ IQR)	40.0 (39.7/40.4)	40.1 (39.0/40.9)	.77†
Birth weight (g), median ($\pm$ IQR)	3555 (3305/3865)	3575 (3220/3820)	.99†
Birth length (cm), median ($\pm$ IQR)	52 (50/53)	52 (50/53)	.96†
Siblings, no. (%)			
0	30 (50)	10 (45.4)	
1	19 (31.7)	4 (18.2)	
2	5 (8.3)	5 (22.7)	
3	4 (6.7)	3 (13.6)	
4	2 (3.3)	0 (0)	.24

Values in boldface are significant.

IQR, Interquartile range.

*Comparison between the farming and nonfarming groups.

†Wilcoxon rank sum test; otherwise χ^2 test.

plasmid standard. Each sample was analyzed in triplicate with a Light-Cycler 480 System.

Statistical analysis

Data were generally not normally distributed, and not all data could be transformed to normality. Nondetectable cytokine concentrations were assigned a value of 0.01 pg/mL for inclusion into the analysis. Nonparametric tests (Mann-Whitney and Wilcoxon tests) were used to compare the median of gene expression, proliferation, and cytokine concentrations, and parametric tests were used for normally distributed data (surface expression). Data were reported as the mean $\pm$ SEM or the median $\pm$ interquartile range, depending on the distribution. Treg cell suppression was assessed by comparing multiple groups/2 groups with ANOVA and *t* tests. 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 and logistic regression analyses were calculated and included potential confounders, such as maternal smoking and education. Odds ratios were reported depending on regression analysis. Statistical significance was defined as a *P* value of less than .05. Data analysis was performed with SAS 9.1 (SAS Institute, Inc, Chicago, Ill) and SigmaStat 3.5 (Systat Software, Inc, San Jose, Calif) software.

RESULTS

Population characteristics

Table I shows the characteristics of 82 included subjects (22 neonates of farming mothers and 60 of nonfarming mothers). Farming mothers smoked less and showed a trend toward less education compared with nonfarming mothers (*P* = .04 and .06,

respectively). Both factors were tested for potential confounding but were revealed not to be confounders. Maternal atopy was less in farming mothers, although not significantly.

Treg cell numbers are quantitatively and qualitatively increased in cord blood of farming mothers

We assessed Treg cells by measuring the surface expression of CD4⁺CD25^{high} T cells (as a percentage of CBMCs) in parallel expressing FOXP3 and low CD127 levels and examining Treg cell markers, including FOXP3, GITR, and LAG3 at the mRNA level. Treg cell markers were increased after PHA and Ppg stimulation (*P* < .05, data not shown). Comparing the cord blood of farming versus nonfarming mothers, CD4⁺CD25^{high} T cells, FOXP3, and trendwise LAG3 mRNA, but not GITR, levels were increased in offspring of farming mothers before or after PHA, Der p 1 + Ppg, or Ppg stimulation, respectively (Fig 1 and see the Results section in this article's Online Repository at www.jacionline.org). Treg cells are known to be increased in cord blood of nonatopic compared with atopic mothers.¹⁷ We detected higher Treg cell counts in CBMCs (both unstimulated and PHA stimulated) of nonatopic farming mothers compared with those of nonfarming mothers. We next tested whether Treg cells of farm children were also functionally more efficient by assessing their suppressive capacity. Only nonatopic donors were chosen. Indeed, the cord blood of farm children showed more efficient

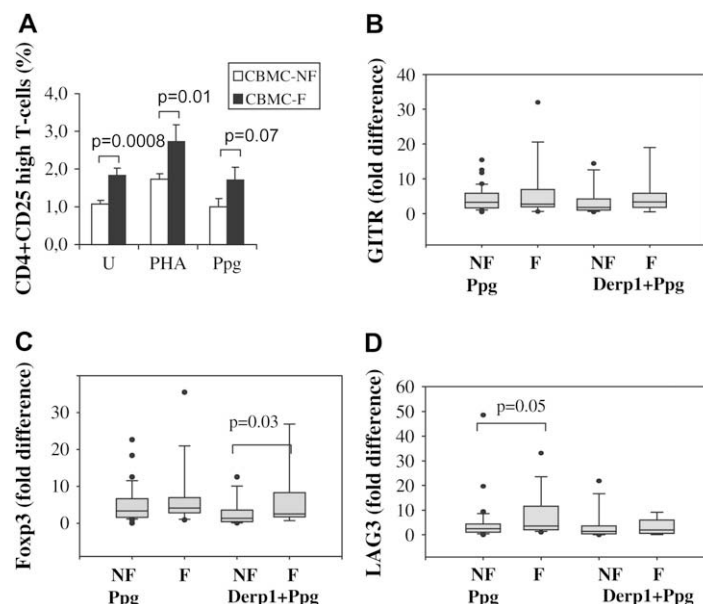


FIG 1. The percentage of CD4⁺CD25^{high} T cells (**A**) and expression of Treg cell-associated genes (**B-D**) in CBMCs of offspring of nonfarming and farming mothers. The *t* test was used (mean $\pm$ SEM) for surface expression data, and the Mann-Whitney rank sum test was used for gene expression data (median $\pm$ interquartile range and outliers). The mRNA expression was presented as the fold difference of stimulated/unstimulated samples relative to the housekeeping gene 18S (*n* = 44 for CBMCs from the offspring of nonfarming mothers; *n* = 19 for CBMCs from the offspring of farming). NF, Nonfarming; F, farming; U, unstimulated.

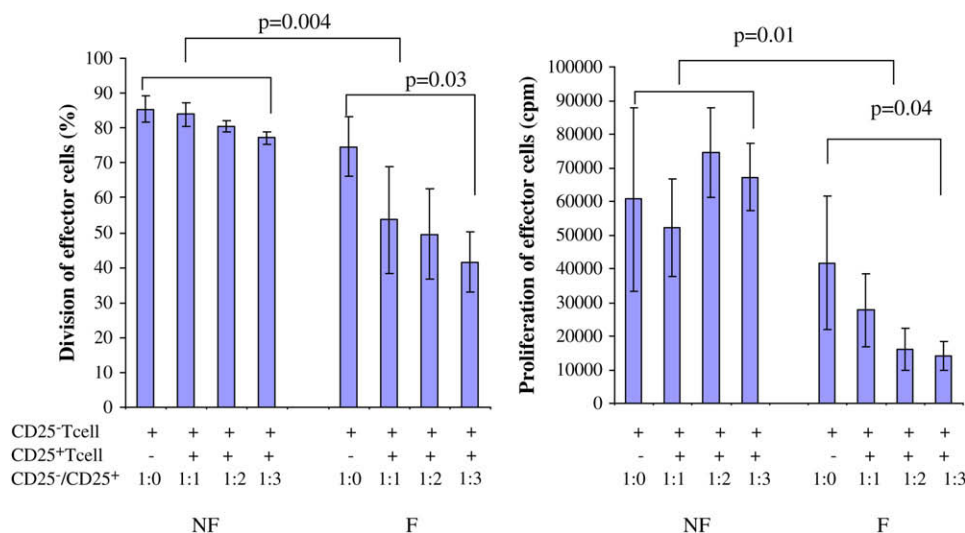


FIG 2. The suppressive capacity of CD4⁺CD25⁺ Treg cells on CD4⁺CD25⁻ T effector cells in CBMCs of offspring of nonfarming mothers (NF) and offspring of farming mothers (F). CD4⁺CD25⁻ T effector cells (20,000 cells/well) labeled with CFSE, cultured with irradiated CD3⁻ cells (40,000 cells/well), and stimulated with PHA (0.8 μ g/mL), with or without CD4⁺CD25⁺ Treg cells (20,000/40,000/60,000 cells/well). After 72 hours of culture, the percentage of newly divided CD4⁺CD25⁻ T effector cells was measured with FACSscan, and proliferation was assessed based on tritiated thymidine incorporation (*n* = 7 nonfarming mothers/*n* = 7 farming mothers). Comparison between groups was performed with ANOVA and the *t* test (see the Methods section).

suppression of effector T-cell division (*P* = .004, Fig 2 and see the Results section in this article's Online Repository).

Different farm exposures showed heterogeneous effects on immune regulation

To disentangle the specific stimuli in the farming environment, we examined the effect of exposures to stables and barns, the

number of animal species, and maternal milk intake on immune regulation (Table II). For analysis of distinct exposures, all mothers with exposure were assessed, and this included some nonfarming mothers. These did not work or live on farms but visited their neighbors on farms and thus had some exposure to stables, barns, and animals or drank farm milk during pregnancy. Although farming had an effect on several Treg cell markers, including function, distinct exposures (different sample sizes) had different effects. Exposure

TABLE II. Association of maternal exposure in pregnancy and immunologic parameters in cord blood of their offspring

Variable	Stable (n = 33/82), yes		Barn (n = 20*/82), yes		Farm milk (n = 20*/82), yes		No. of animal species (0-1, ≥ 2 ; n = 40/82 (≥ 2))	
	Median	P value	Median	P value	Median	P value	Median	P value
Treg cell markers								
CD4 ⁺ CD25 ^{high} U (%)								
No	1.2	.08	1.3	.53	1.2	.09	1.2	.48
Yes	1.6		2.0		1.6		1.7	
CD4 ⁺ CD25 ^{high} PHA (%)								
No	1.7	.10	1.9	.83	1.9	.37	1.9	.92
Yes	2.0		1.9		2.0		1.9	
CD4 ⁺ CD25 ^{high} Ppg (%)								
No	0.5	.01	1.0	.37	1.1	.65	1.0	.48
Yes	1.5		1.4		1.4		1.3	
FOXP3 Der p 1 + Ppg (fd)								
No	1.4	.17	1.6	.09	1.4	.06	1.4	.04
Yes	2.5		4.3		2.7		2.9	
GITR PHA (fd)								
No	4.0	.37	4.0	.13	4.0	.65	4.0	.03
Yes	7.7		8.3		7.3		11.7	
LAG3 Ppg (fd)								
No	2.7	.21	2.4	.11	2.6	.11	2.3	.38
Yes	3.3		3.7		3.7		3.1	
Cytokines								
IL-10 U								
No	0.01	.07	0.01	.26	0.01	.09	0.01	.05
Yes	0.08		0.08		0.10		0.09	
IFN- γ U								
No	0.08	.60	0.08	.26	0.45	.10	0.01	.33
Yes	0.01		0.01		0.01		0.77	
IFN- γ Der p 1								
No	47.7	.10	48.6	.07	47.7	.10	46.4	.002
Yes	81.1		88.2		74.4		97.8	
IFN- γ Ppg								
No	47.0	.21	50.2	.06	47.8	.10	46.3	.02
Yes	69.0		95.1		74.4		97.8	
IL-13 Der p 1 + Ppg								
No	52.8	.12	51.8	.07	43.2	.55	42.2	.57
Yes	27.9		25.2		28.5		34.8	
IL-5 Der p 1 + Ppg								
No	49.2	.04	48.8	.10	40.1	.13	48.9	.05
Yes	31.9		30.6		38.4		28.6	

Bivariate analysis for each exposure is shown separately (median and *P* values). Values are shown for exposure to stables, barns, and farm milk during pregnancy. Cytokines are shown in picograms per milliliter, and gene expression of FOXP3, GITR, and LAG3 as is shown as a fold difference (*fd*). *P* values were determined with the Wilcoxon test. Values in boldface are significant.

U, Unstimulated.

*These are not identical subjects for barn and farm milk.

to stables showed an increase in CD4⁺CD25^{high} cell counts (Ppg stimulated) and lower IL-5 levels (Der p 1 + Ppg stimulated). Increased CD4⁺CD25^{high} expression (Ppg stimulated) was correlated with lower IL-5 levels ($r = .61$), although this was not significant ($P = .15$, data not shown). Exposure to barns showed nonsignificant effects on T_H1 cytokines. A higher number of animal species increased Treg cell markers (FOXP3 Der p 1 + Ppg, GITR, and PHA compared with unstimulated expression) and resulted in higher T_H1 expression (IFN- γ , Der p 1 and Ppg) and a trend toward lower T_H2 expression (IL-5, Der p 1 + Ppg). Comparing 3 groups of nonfarming nonexposed, nonfarming exposed to milk or animals, and farming mothers, primarily IFN- γ (Ppg and Der p 1 stimulated) levels were highest in the nonfarming exposed group ($P = .04$, see Table E1 in this article's Online Repository at www.jacionline.org).

To further investigate whether the different exposure had an effect on epigenetic regulation of FOXP3 expression, we examined the relevance of exposure on the demethylation of an evolutionarily conserved element within the *FOXP3* locus (TSDR) in a subset of 59 subjects (Table III). The selected group was similar to the whole group, excluding a selection bias. This locus had previously been shown by us to be selectively demethylated only in stably *FOXP3*-expressing natural Treg cells but not in transiently *FOXP3*-expressing and recently activated effector T cells.^{20,21} Consistently, the amount of demethylated TSDR was higher with any single exposure compared with that seen after no exposure, although significantly only for maternal intake of farm milk during pregnancy ($P = .02$). When analyzing subjects with any exposure compared with no exposure, a similar trend was observed ($P = .09$).

TABLE III. *FOXP3* demethylation (as a percentage) in cord blood in relation to different farming exposures

	No.	Minimum	1st quartile	Median	3rd quartile	Maximum	<i>P</i> value (Wilcoxon)
Farm child							
No	39	0.6	1.2	1.6	2.1	3.3	.38
Yes	20	0.8	1.5	1.8	2.3	3.0	
Preg stable							
No	34	0.6	1.3	1.6	2.0	3.1	.25
Yes	25	0.8	1.4	1.8	2.3	3.3	
Preg barn							
No	43	0.6	1.2	1.6	2.1	3.3	.21
Yes	16	0.9	1.6	1.8	2.4	3.0	
Preg farm milk							
No	40	0.6	1.3	1.6	1.9	3.1	.02
Yes	19	0.8	1.6	2.0	2.4	3.3	
No. of animal species							
0-1	43	0.6	1.3	1.6	2.1	3.1	.41
≥2	16	0.9	1.5	1.8	2.3	3.3	
Any exposure							
No	32	0.6	1.2	1.6	1.9	3.1	.09
Yes	27	0.8	1.4	1.8	2.3	3.3	

Bivariate analysis for each exposure is shown separately (minimum, quartiles, median, maximum, and *P* values). Values in boldface are significant.

Preg, Exposure during pregnancy.

Lower T_H2 cytokine levels and proliferation after Der p 1 + Ppg stimulation in offspring of farming mothers

Next we aimed to investigate whether farm exposure resulted in lower lymphocyte proliferation and decreased T_H2 (proallergic) cytokine levels. Indeed, proliferation of offspring from farming mothers was lower compared with that seen in offspring from nonfarming mothers ($P = .04$, Table IV). Regarding overall T-cell cytokine levels, there was no significant difference in either group (Table IV and see Table E2 in this article's Online Repository at www.jacionline.org). However, levels of the proallergic T_H2 cytokines IL-5 and IL-13 were significantly or trendwise decreased in offspring of farming compared with nonfarming mothers (Der p 1 + Ppg stimulation). IL-6 levels were higher in offspring of farming mothers (Der p 1 + Ppg stimulation, $P = .01$; Der p 1 stimulation, $P = .05$; see the Results section in the article's Online Repository). Of note, subset analysis revealed that IL-6 and IL-5 were expressed by CD4/CD8 T cells and, at a low percentage, by natural killer cells, and IL-13 was expressed primarily by CD4/CD8 T cells and CD3⁺ cells (see the Results section in this article's Online Repository).

When correlating Treg cell markers with T_H1/T_H2 cytokines, in offspring of farming mothers, cord blood FOXP3 expression was positively correlated with higher IFN- γ levels after Ppg or Ppg + Der p 1 stimulation ($r = 0.51$, $P = .047$ and $r = .81$, $P = .01$) and negatively correlated with IL-13 or IL-5 expression after Ppg or Ppg + Der p 1 stimulation, respectively ($r = -0.72$, $P = .02$ and $r = -0.52$, $P = .18$). In CBMCs from offspring of nonfarming mothers, no significant correlations were observed apart from FOXP3/IL-13 expression being negatively correlated after Ppg stimulation ($r = -0.47$, $P = .002$).

Correlation between IL-17 and IFN- γ

A T-cell population closely linked to Treg cells, namely T_H17 cells, can also influence T_H1/T_H2 responses.²² IL-17 secretion was undetectable in unstimulated and PHA-stimulated samples (Table IV). However, after innate and allergen stimulation,

IL-17 secretion was weakly increased, although not significantly different, between the 2 groups. The correlation of IL-17 and IL-13 secretion (T_H2) was positive after Ppg stimulation ($r = 0.75/0.79$) independent of farming (Table V). However, the correlation between IL-17 and IFN- γ (T_H1) was significantly positive ($r > 0.6$), although only among offspring of farming mothers after PHA/Ppg stimulation.

DISCUSSION

This study suggests a mechanism by which the protective effect of farming exposure in pregnancy might influence neonatal immune development and subsequently reduce the development of allergic diseases. Treg cells, comprehensively assessed in number, gene expression, epigenetic regulation, and function, were mainly at higher levels and more efficient in offspring of farming compared with nonfarming mothers after specific stimulation. This was associated with decreased T_H2 cytokine levels and less proliferation in offspring of farming mothers. Exposure to several animal species had an effect on Treg cell markers and IFN- γ secretion, whereas exposure to stables and milk intake showed heterogeneous effects with an incomplete overlap, suggesting diverse additive effects. In addition, T_H17 cells were regulated independently of farming status but were positively correlated with T_H1 lineages, primarily in offspring of farming mothers.

Several farming studies have reproduced the finding that farming exposure is protective against the development of atopic disease.^{1,2} Two factors seem to be particularly important for this effect. First, early prenatal influences on immune modulation have the strongest effect on reducing later atopic development, and second, microbial exposure has been identified as a crucial "entity of allergy-protective exposure."^{1,2} These observations suggest that influences of the environment modulate this very sensitive period of fetal immune development in such a strong way³ that it results in long-term immune effects, either through direct or indirect effects on the maternal immune milieu, maternal-fetal immune interactions, or quite likely a combination of both. The

TABLE IV. Lymphocyte proliferation and cytokine concentrations in CBMCs of offspring of nonfarming and farming mothers after various stimulations

Parameter	Stimuli	Nonfarming CBMCs, median $\pm$ IQR (n = 59)	Farming CBMCs, median $\pm$ IQR (n = 21)	P value*
LP	U	3,800 (2,866/5,973)	3,562 (2,201/6,268)	.47
	PHA	61,573 (43,624/9,4685)	64,894 (44,357/91,877)	.96
	Ppg	7,591 (4,759/10,659)	4,423 (2,899/8,080)	.04
	Der p 1 + Ppg	7,899 (6,090/11,424)	3,830 (2,491/7,298)	.04
IFN- γ	U	0.1 (0.01/1.8)	0.01 (0.01/0.8)	.27
	PHA	58 (17.7/100)	76 (29/202)	.31
	Ppg	50 (31/119)	74 (33/128)	.36
	Der p 1 + Ppg	107 (54/155)	120 (52/157)	.71
IL-13	U	0.33 (0.01/0.8)	0.2 (0.2/0.6)	.97
	PHA	93 (4/734)	31 (7/2013)	.99
	Ppg	37 (18/59)	22 (13/67)	.70
	Der p 1 + Ppg	52 (28/64)	25 (16/41)	.10
IL-5	U	0.01 (0.01/0.02)	0.01 (0.01/0.1)	.61
	PHA	18 (3/36)	8 (3/36)	.85
	Ppg	39 (19/74)	25 (15/50)	.17
	Der p 1 + Ppg	49 (32/106)	23 (16/39)	.02
IL-10	U	0.01 (0.01/0.1)	0.1 (0.01/0.1)	.09
	PHA	79 (25/130)	60 (30/151)	.89
	Ppg	1,053 (772/1,582)	1,030 (802/1,490)	.58
	Der p 1 + Ppg	1,978 (1,251/2,516)	1,627 (1,115/2,469)	.50
IL-6	U	3.6 (1.3/14.0)	4.8 (1.9/26.9)	.60
	PHA	26,028 (17,620/35,433)	34,000 (21,876/41,998)	.25
	Ppg	29,893 (25,205/43,876)	34,000 (26,453/51,814)	.61
	Der p 1 + Ppg	34,100 (22,575/40,967)	64,536 (34,000/11,1784)	.01
IL-17	U	0.01 (0.01/0.4)	0.01 (0.01/0.5)	.97
	PHA	0.01 (0.01/0.2)	0.1 (0.01/0.7)	.25
	Ppg	3.3 (1.2/8.8)	1.5 (1.1/8.8)	.35
	Der p 1 + Ppg	5.0 (1.4/7.9)	2.7 (1.7/9.8)	.39

Values in boldface are significant.

IQR, Interquartile range, LP, lymphocyte proliferation; U, unstimulated.

*Comparison between nonfarming and farming groups (Wilcoxon rank sum test). Lymphocyte proliferation values are shown in counts per minute (median $\pm$ interquartile range), and cytokine values are shown in picograms per milliliter.

TABLE V. Correlation between IL-17 and T_H2/T_H1 or IL-10 secretion

Cytokines (pg/mL)	Stimuli	Nonfarming CBMCs (CBMC-NF), n = 59		Farming CBMCs (CBMC-F), n = 18	
		r	P value	r	P value
IL-17/IL-13	PHA	0.10	.46	0.55	.03
	Ppg	0.75	<.0001	0.79	.0001
IL-17/IFN- γ	PHA	0.13	.34	0.83	.0001
	Ppg	0.26	.05	0.61	.01
IL-17/IL-10	PHA	0.24	.08	0.41	.11
	Ppg	-0.09	.51	0.23	.37

Analysis with the Spearman correlation coefficient (r). Values in boldface are significant.

early long-lasting immune effect is supported by the increase of innate receptors, including TLR2 and CD14, at school age, particularly when exposure to stables was present in pregnancy.³ Although it is remarkable that effects *in utero* are capable of inducing a long-lasting immune modulation, the underlying mechanism is not understood.

Our study potentially adds a novel immunoregulatory mechanism by which maternal exposure to this natural model of allergy protection can modulate Treg cells already before birth, indirectly reflecting early immune development. The data suggest that Treg cells assessed in terms of numbers and gene expression might be functionally more efficient in early immune modulation in offspring of farming mothers. In addition, data on *FOXP3* demethylation,

primarily for milk exposure, point also in this direction. Although the trendwise difference in this study requires confirmation, this novel epigenetic candidate suggests early epigenetic immune regulation through specific stimulation during pregnancy.

Importantly, the Treg cell effects in this study were not only associated with more efficient functional suppression in the context of farm exposure but were also associated with effector T-cell modulation, such as a decrease in levels of the T_H2 cytokine IL-5. This shift to an anti-T_H2 allergy-protective immune pattern was present at birth but requires specific stimulatory conditions. We chose the stimulatory conditions to mimic a part of natural farm exposure using either innate stimulation (Ppg) or a combination with allergen, which might in fact reflect *in vivo* exposures of

the farming environment more closely than one single experimental stimulus, which does not occur in real life. Indeed, stimulation with Ppg in combination with allergen showed the strongest effects. Of note, LPS content in our reagents was low and did not influence the findings.

Murine neonatal data support the hypothesis that appropriate exposure in early life is required to prevent airway allergic inflammation. Wang and McCusker²³ have demonstrated that prevention of airway allergy might be best achieved by specific exposure (LPS and allergen) of the airway mucosa early in life to environmental antigens, potentially reflecting early mucosal tolerance. Consequently, one might speculate that subsequent development of allergic diseases results from an early immune deficiency to respond adequately to specific stimuli caused by a lack of exposure, in our case microbial farm exposure.

Regarding the type of microbial exposure, exposure to an increasing number of animal species might potentially indicate an incremental dose-response effect, contributing to more pronounced immune stimulation early in life. Although we could not disentangle one specific exposure as indicative of Treg cell induction, distinct exposures might reflect exposure to a variety of microbes, reflecting nonspecific stimulation. Intriguingly, some effects were not restricted to farming mothers. Offspring of nonfarming mothers, who did not live and work on farms but were occasionally exposed to farm animals or farm milk, showed, for example, higher IFN- γ concentrations than any other group. Although the overall effects are still strongest in the offspring of farming mothers, these data, although in small numbers, suggest a potential immunomodulatory effect present for even noncontinuous exposure.

Although T_H2 responses were decreased in offspring of farm-exposed mothers, one might expect either a parallel decrease in T_H1 cytokine levels through general suppression or an additional increase in T_H1 cytokine levels reflecting selective regulation. Animal exposure showed an increased T_H1 response, but overall farming did not. This might be explained by the requirement of a certain level of stimulation, no direct effect on T_H1 lineage, or more complex regulatory features than T_H2/T_H1 pathways in overall farming. Supporting the latter, promising candidates are T_H17 cells, which we previously demonstrated to be immature at birth compared with adult immune responses.²⁴ Thus no significant differences between farm and nonfarm children might be due to low IL-17 secretion at this early stage of maturation. However, the positive correlation primarily with T_H1 cells in the context of farming might account for a role of T_H17 cells in balancing the immune system during maturation in close association with T_H1 lineages. In parallel with our study, T_H17/T_H1 correlations were also described in patients with Crohn disease.²⁵ Although T_H17 cells are shown to be involved in atopic dermatitis,²⁶ they are a promising candidate in the regulation of atopic diseases. However, these hypotheses have to be confirmed in larger studies.

Finally, one fascinating speculation is that 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. In conventional immunotherapy strategies, a protective effect might be mediated through Treg cells.^{6,27,28} This hypothesis clearly requires further investigation but would offer some consideration for future preventive strategies.

In summary, this study proposes a novel mechanism by which maternal farm exposure modulates offspring immune responses. Treg cells in cord blood were increased in number and function in

this unique model of *in vivo* allergy protection and resulted in suppression of proallergic immune responses, such as T_H2 cytokines associated with lower lymphoproliferation. This regulatory T-cell population keeping healthy immune responses in balance and thus preventing disease development might have the potential to contribute to the long-lasting allergy-protective effect of Treg cells in older children and adults.^{13,23,29-32} However, only a future follow-up of these children until the age of 6 years can determine whether this neonatal immunologic starting condition is able to shape the immune regulation in such a way to truly protect against the development of atopic disease.

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Key messages

- Farm exposures during pregnancy increase Treg cell numbers and function.
- Maternal farm exposure might reflect a natural model of immunomodulation, shaping a child's immune system in early life.

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METHODS

Study population

The laboratory investigators were blinded to clinical information.

Lymphocyte proliferation and cytokine secretion of CBMCs

Cord blood was collected from the umbilical vein after delivery and processed within 24 hours. CBMCs were isolated by means of density-gradient centrifugation with Ficoll-Hypaque Plus (Amersham Bioscience, Uppsala, Sweden) after dilution in PBS (Gibco, Invitrogen). Cells washed in RPMI 1640 were diluted in 10% human serum (Sigma-Aldrich, Seelze, Germany) to a concentration of 5×10^6 cells/mL. For lymphocyte proliferation, 0.5×10^6 cells/well CBMCs were stimulated in replicates with Ppg (10 μ g/mL, *Staphylococcus aureus*), PHA (5 μ g/mL; all Sigma-Aldrich), and Der p 1 (30 μ g/mL; Indoor Biotechnologies, Charlottesville, Va) or a combination of Der p 1 and Ppg for 3 days and compared with unstimulated cells. Endotoxin concentrations in Ppg, Der p 1, and PHA, as measured with the Limulus assay, were low (<0.01 EU/mL = 0.002 ng/mL) and did not significantly change lymphocyte proliferation or cytokine secretion. By testing the functional ability of CBMCs with different doses of LPS and active components, such as Lipid A (0.01–100 ng/mL), we detected increased lymphocyte proliferation with doses of greater than 1 ng/mL, and therefore levels of less than 0.01 ng/mL had no influence on the analysis. After incubation at 37°C in a humidified 5% CO₂ chamber, samples were pulsed with 1 μ Ci of tritiated thymidine for an additional 8 hours. Cells, harvested with a ComBI cell harvester (Skatron Instruments, Lier, Norway) onto filter plates, were read with a β counter. Proliferation was assessed as counts per minute and quantified by using the stimulation index, which was calculated as the ratio of mean counts per minute of stimulated over unstimulated replicates.

For cytokines, the lower limit of detection of the assay was as follows: IL-2, 1.1 pg/mL; IL-4, 0.4 pg/mL; IL-5, 1.8 pg/mL; IL-6, 0.5 pg/mL; TNF- α , 3.0 pg/mL; IL-10, 0.9 pg/mL; IL-13, 2.1 pg/mL; IL-17, 0.2 pg/mL; IFN- γ , 1.3 pg/mL; IL-12, 0.5 pg/mL; and GM-CSF, 1.0 pg/mL.

Flow cytometry

For surface staining, CBMCs were incubated in aliquots of 5×10^5 cells (100 μ L 1 $\times$ PBS, 5% FCS, and 0.5% Tween) with saturating concentrations of fluorochrome-labeled antibodies for 30 minutes at 4°C. The samples were washed twice with 1 mL of buffer and resuspended in 400 μ L of PBS-FCS. For intracellular FOXP3 staining, cells were stained with 8 μ L of anti-human CD4-FITC and 4 μ L of anti-human CD25-RPE-Cy5 antibodies per tube (1×10^5 /100 mL). After resuspension, 1 mL of freshly prepared Fixation/Permeabilization working solution buffer (eBioscience NatuTec, Frankfurt, Germany) was added and incubated at 4°C for 60 minutes in the dark. After centrifugation, cells were washed with 1 $\times$ Permeabilization Buffer. After blockade with 2% normal rat serum (2 μ L) in 1 $\times$ Permeabilization Buffer at 4°C for 15 minutes, 10 μ L of anti-human FOXP3-PE antibody or 5 μ L of mouse IgG2a-PE isotype control in 1 $\times$ Permeabilization Buffer was added and incubated at 4°C for 30 minutes in the dark. After a further washing step, cells were resuspended, and samples were analyzed on the cytometer.

Functional analysis of Treg cells

After isolation of CD3⁺ cells with the CD3 isolation kit (Miltenyi Biotec), CD3⁺ cells were irradiated at 30 Gy (3000 rad for 10 minutes). CD3⁺ cell purity reached 98%. CD4⁺CD25[−]/CD4⁺CD25⁺ T cells were isolated in a 2-step procedure by using depletion of non-CD4⁺ cells, followed by positive selection of CD4⁺CD25⁺ T cells (CD4CD25 isolation kit, Miltenyi Biotec). The purity of CD4⁺CD25[−]/CD25⁺ T cells was greater than 95%. CD4⁺CD25[−] T cells were labeled with 5 μ mol/L CFSE (CellTrace CFSE-Cell Proliferation-Kit C34554, Invitrogen) at a final concentration of 1×10^6 cells/mL. CD4⁺CD25[−] T cells (2×10^4 cells/well) were incubated with irradiated CD3⁺ cells (4×10^4 cells/well) in coculture with or without CD4⁺CD25⁺ T cells (2×10^4 cells/well). Results were compared before and after stimulation with 0.8 μ g/mL PHA. After 3 days of culture, division of CD4⁺CD25[−] T cells was analyzed with CellQuest software. The proliferation of CD4⁺CD25[−]

T cells was assessed by using tritiated thymidine incorporation. In supernatants cytokine concentrations of IFN- γ , IL-13, IL-5, IL-10, TNF- α , and IL-17 were measured with the Human Cytokine-Multiplex-Assay-Kit (Bio-Rad).

Real-time quantitative RT-PCR

Total RNA (1 μ g) isolated with TRI Reagent was processed with reverse transcriptase (Invitrogen). For RT-PCR, we used 0.34 μ L of cDNA, 7.5 μ L of primer, 9.66 μ L of DEPC water, and 12.5 μ L of SYBR Green Master-Mix/well (total, 30 μ L; Applied Biosystems). Gene-specific PCR products were measured continuously by using the iCycler iQ-multicolor Real-Time PCR-Detection-System (Bio-Rad) for 40 cycles. All experiments were run in duplicates with the same thermal-cycling parameters. Nontemplate controls and dissociation curves were used to detect primer-dimer and nonspecific amplification. PCR products were separated on a 3% agarose gel to control for specificity and the expected size of the PCR fragment. mRNA-specific oligonucleotide primers (forward/Reverse) were designed with Vector NT I-Advance10: 18S, 5'-AGTCCCTGCCCTTTGTACACA-3'/5'-GATCCGAGGGCCTCACTAAAC-3'; FOXP3, 5'-ACCTTCCCAAATCCAGTGC-3'/5'-GAAGATGGTCCGCCTGGC-3'; LAG3, 5'-CAATGGCGACTTTACCC TTC-3'/5'-CCTCTGGGATGGGGTGTG-3'; and GTR, 5'-GCACCACCCTGTCCCCC-3'/5'-CAGCGTTGTGGGTCTGTTC-3'.

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

Genomic DNA was isolated with the Dneasy blood kit (Qiagen). Bisulfite treatment of genomic DNA was performed. Real-time PCR for measurement of the FOXP3 TSDR was performed in a final reaction volume of 20 μ L with the Roche LightCycler 480 Probes Master (Roche Diagnostics) 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), and 30 ng of bisulfite-treated genomic DNA template or respective amount of plasmid standard. Each sample was analyzed in triplicate with a Light-Cycler 480 System (Roche). Cycling conditions consisted of a 95°C preheating step for 10 minutes and 50 cycles of 95°C for 15 seconds, followed by 1 minute at 61°C.

RESULTS

Treg cells were quantitatively and qualitatively increased in cord blood of farming mothers

Assessing Treg cells, CD4⁺CD25^{high} T cells (as a percentage of CBMCs) were increased in CBMCs from farming mothers (before and after PHA and trendwise for Ppg stimulation compared with CBMCs from nonfarming mothers, Fig 1). The percentage of FOXP3⁺ T cells in CD4⁺CD25^{high} T cells was 85% to 100%; 1% to 5% of CD4⁺CD25[−] T cells were FOXP3⁺ cells. In CBMCs from farming mothers, FOXP3 and LAG3 expressions were significantly higher compared with those seen in CBMCs from nonfarming mothers after Der p 1 + Ppg or Ppg stimulation, respectively ($P = .03/.05$). The suppressive capacity of CD4⁺CD25⁺ Treg cells on the division and proliferation of CD4⁺CD25[−] effector T cells showed higher suppression in CBMCs from farming mothers compared with CBMCs from nonfarming mothers (10.3% vs 1.6%/37.8% vs 9.6%, $P = .003/.008$, Fig 3). No significant differences for CD4⁺, CD8⁺, and CD19⁺ cells between CBMCs from farming mothers and CBMCs from nonfarming mothers were detected (not shown).

Lower T_H2 cytokine secretion and proliferation after Der p 1 + Ppg stimulation in offspring of farming mothers

Subset analysis revealed that IL-6 and IL-5 were expressed by CD4/CD8 T cells and, at a low percentage, by natural killer cells, and IL-13 was expressed primarily by CD4/CD8 T cells in addition

to CD3⁺ cells. In detail, IL-5-positive cells in the CBMC population were produced by a low percentage of CD4⁺ T cells (0.6%), CD8⁺ T Cells (0.3%), and natural killer cells (0.4%). IL-6 was produced by a low percentage of CD4⁺ T cells (0.4%), CD8⁺ T Cells (0.4%), natural killer cells (1.4%), and monocytes (0.5%). IL-13 CD4⁺ cells accounted for 3.02% and IL-13 CD8⁺ cells accounted for 1.34% in CBMCs. CD3⁺ cells accounted for 1.91%.

Different farm exposures showed heterogeneous effects on immune regulation

Offspring of nonfarming mothers, who did not live and work on farms but were occasionally exposed to farm animals or farm milk, showed similar effects to offspring of farming mothers.

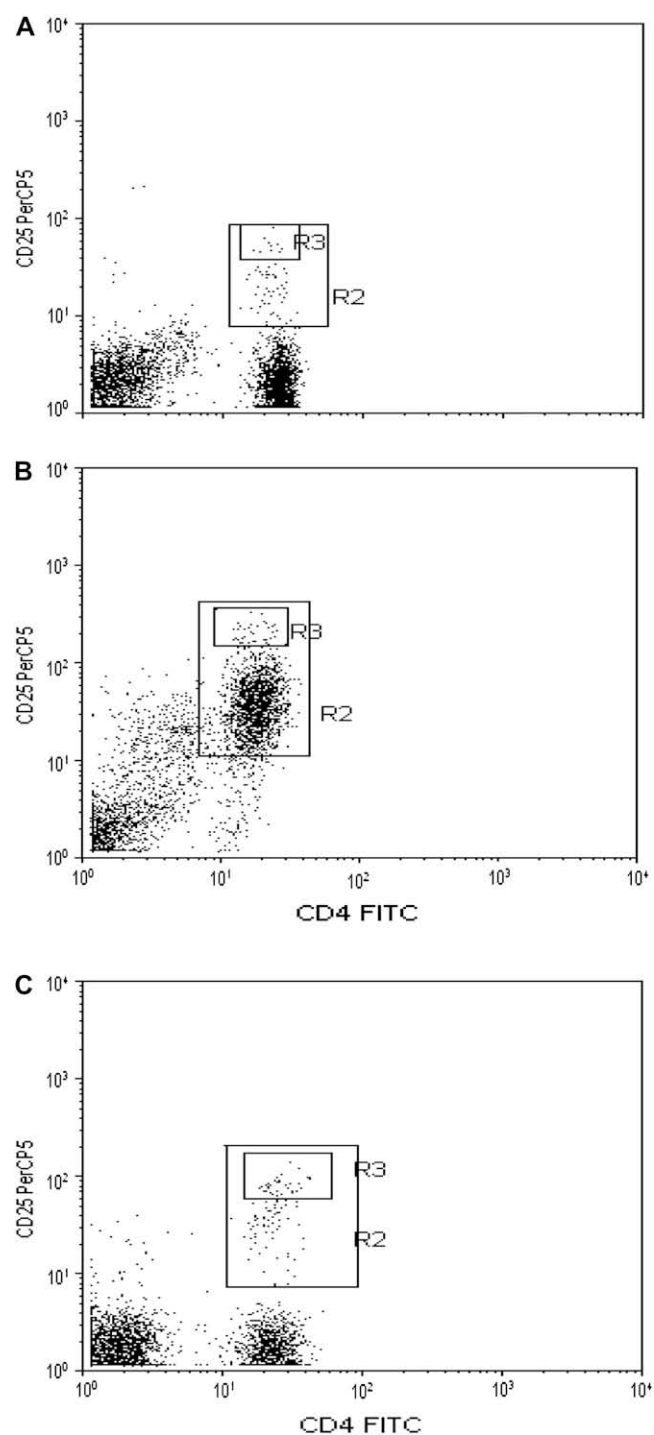


FIG E1. Gating strategy of CD4⁺CD25^{high} T cells after no stimulation (**A**) and stimulation with PHA (**B**) and Ppg (**C**). CD4⁺CD25^{high} T cells are labeled as R3 in the CD4⁺CD25⁺ fraction (R2) of lymphocytes (R1).

TABLE E1. Association of exposed and unexposed non-farming and farming CBMCs and immunologic parameters in cord blood

Parameter	Stimuli	Unexposed nonfarming CBMCs, median ± IQR (n = 42)	Exposed nonfarming CBMCs, median ± IQR (n = 15)	Farming CBMCs, median ± IQR (n = 21)	P value* (Kruskal-Wallis test)	Exposed nonfarming CBMCs milk/animal, median ± IQR (n = 11)	P value* (Kruskal-Wallis test)
CD4	U	1.2 (1.0/1.3)	1.0 (0.7/1.3)	2.1 (1.5/2.2)	.01	1.2 (0.6/1.4)	.01
CD25	PHA	1.9 (1.2/2.2)	1.7 (1.6/2.0)	2.4 (1.8/3.5)	.12	1.6 (1.6/1.8)	.08
high	Ppg	0.6 (0.4/1.1)	1.3 (0.7/2.0)	1.6 (1.0/2.4)	.10	1.1 (0.4/2.5)	.16
FOXP3 (fd)	Der p 1 + Ppg	1.4 (0.4/1.9)	6.6 (0.6/12.6)	2.7 (2.0/8.3)	.11	6.6 (0.6/12.6)	.11
LP	PHA	58,194 (40,828/88,520)	89,411 (70,723/11,7775)	64,894 (44,357/91,877)	.03	100,408 (89,411/14,4971)	.004
	Ppg	7,591 (4,629/10,120)	7,899 (4,761/10,658)	4,423 (2,899/8,080)	.10	9,917 (6,678/12,974)	.07
	Der p 1 + Ppg	8,669 (5,589/11,015)	7,773 (6,961/11,423)	3,830 (2,491/7,298)	.11	7,773 (6,961/11,423)	.11
IFN-γ	PHA	50 (15/97)	81 (59/198)	76 (29/202)	.13	81 (58/266)	.12
	Ppg	46 (24/119)	69 (56/121)	74 (33/128)	.16	97 (59/132)	.04
	Der p 1	46 (19/98)	72 (52/115)	76 (12/110)	.12	81 (54/117)	.04
IL-13	Der p 1 + Ppg	42 (28/64)	57 (36/83)	25 (16/41)	.25	57 (36/83)	.25
IL-5	Der p 1 + Ppg	40 (28/132)	49 (33/68)	23 (16/39)	.07	49 (34/ 68)	.07
IL-6	Der p 1 + Ppg	34,000 (22,575/40,706)	36,616 (26,901/47,763)	64,536 (34,000/111,784)	.04	36,616 (26,901/47,763)	.04

The group of CBMCs from offspring of exposed nonfarming mothers was exposed to stables, barns, or farm milk or more than 2 animal species. The group of CBMCs from offspring of exposed (milk/animals) nonfarming mothers was exposed to either farm milk or 2 or more animal species. The rightmost *P* value compares CBMCs from offspring of exposed nonfarming mothers (milk/animal) with CBMCs from offspring of unexposed nonfarming and farming mothers.

IQR, Interquartile range.

TABLE E2. Cytokine concentrations in CBMCs of offspring of nonfarming and farming mothers

Parameter	Stimuli	Nonfarming CBMCs (median ± IQR (n = 59))	Farming CBMCs, median ± IQR (n = 21)	P value*
LP (cpm)	U	3,800 (2,866/5,973)	3,563 (2,202/6,268)	.47
	PHA	61,573 (43,624/94,685)	64,895 (44,357/91,877)	.96
	Ppg	7,592 (4,759/10,659)	4,423 (2,899/8,080)	.04
	Der p 1	5,000 (3,366/7,515)	3,171 (2,171/8,000)	.15
	Der p 1 + Ppg	7899 (6090/11424)	3,830 (2,491/7,298)	.04
IFN-γ	U	0.1 (0.01/1.8)	0.01 (0.01/0.8)	.27
	PHA	59 (18/100)	76 (29/202)	.31
	Ppg	50 (31/119)	74 (33/128)	.36
	Der p 1	51 (25/101)	76 (12/111)	.44
	Der p 1 + Ppg	107 (53/155)	120 (52/157)	.71
IL-13	U	0.33 (0.01/0.8)	0.2 (0.2/0.6)	.97
	PHA	93 (4/734)	31 (7/2013)	.99
	Ppg	37 (18/59)	22 (13/67)	.70
	Der p 1	10 (5/19)	12 (2/18)	.58
	Der p 1 + Ppg	52 (28/64)	25 (16/41)	.10
IL-5	U	0.01 (0.01/0.02)	0.01 (0.01/0.1)	.61
	PHA	18 (3/36)	8 (3/36)	.85
	Ppg	39 (19/74)	25 (15/50)	.17
	Der p 1	11 (5/107)	6 (3/11)	.20
	Der p 1 + Ppg	49 (32/106)	23 (16/39)	.02
IL-10	U	0.01 (0.01/0.1)	0.01 (0.01/0.1)	.09
	PHA	79 (25/130)	60 (30/151)	.89
	Ppg	1,053 (772/1,582)	1,030 (802/1,490)	.58
	Der p 1	193 (98/262)	159 (81/245)	.71
	Der p 1 + Ppg	1,978 (1,251/2,516)	1,627 (1,115/2,469)	.50
IL-6	U	3.6 (1.3/14.0)	4.8 (1.9/26.9)	.60
	PHA	26,028 (17,620/35,433)	34,000 (21,876/41,998)	.25
	Ppg	29,893 (25,205/43,876)	34,000 (26,453/51,814)	.61
	Der p 1	35,973 (25,774/49,681)	48,233 (39,087/71,690)	.05
	Der p 1 + Ppg	34,100 (22,575/40,967)	64,536 (34,000/111,784)	.01
IL-17	U	0.01 (0.01/0.4)	0.01 (0.01/0.5)	.97
	PHA	0.01 (0.01/0.2)	0.1 (0.01/0.7)	.25
	Ppg	3.3 (1.2/8.8)	1.5 (1.1/8.8)	.35
	Der p 1	3.2 (0.7/6.0)	0.5 (0.02/5.5)	.23
	Der p 1 + Ppg	5.0 (1.4/7.9)	2.7 (1.7/9.8)	.39
GM-CSF	U	0.01 (0.01/5.0)	0.01 (0.01/0.01)	.09
	PHA	2.8 (0.01/13.0)	0.01 (0.01/92)	.68
	Ppg	111 (10/361)	131 (9/522)	.91
	Der p 1	12 (0.5/97)	33 (0.3/122)	.55
	Der p 1 + Ppg	567 (154/932)	918 (341/2196)	.43
TNF-α	U	0.01 (0.01/0.6)	0.01 (0.01/0.46)	.42
	PHA	141 (25/422)	115 (34/583)	.96
	Ppg	1964 (1272/2981)	1805 (1287/2454)	.43
	Der p 1	778 (374/1612)	925 (419/1152)	.92
	Der p 1 + Ppg	2263 (1823/6627)	3096 (1804/5634)	.93

IQR, Interquartile range; U, unstimulated.

*Comparison between CBMCs from offspring of nonfarming and farming mothers (Wilcoxon rank sum test).