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

TABLE I. Description of analyzed SNPs in T_H17 pathway genes and their respective rs numbers, position in gene structure, and allele frequency

Gene	rs no.	Position to first ATG*	Position in gene structure	Major/minor allele (CEU)†	MAF (CEU)	MAF (PAULCHEN)
RORC	rs949969	11427 (transcript variant 1) 5599 (transcript variant 2)	Intron	C/T	0.25	0.28
	rs7540530	13383 (transcript variant 1) 7555 (transcript variant 2)	Intron	T/C	0.45	0.53
IL23R	rs790631	43119	Intron	T/C	0.25	0.21
	rs7517847	47866	Intron	T/G	0.45	0.60
	rs10889675	88413	Intron	C/A	0.15	0.14
IL17	rs9395766	−5215	5′ Upstream	T/G	0.39	0.37
	rs2275913	−197	Promoter region	G/A	0.36	0.38

*Based on the gene sequences obtained from Ensembl (http://www.ensembl.org; NCBI36 release 54, May 2009).
†*CEU*, Utah residents with Northern and Western European ancestry from the CEPH collection. Data were obtained from the SNPPER database (http://snpper.chip.org/).

analyzed at the mRNA level. Furthermore, cytokine secretion, including T_H17, Treg, T_H1, and T_H2 cytokines, was assessed at the protein level. A recessive model assessed the results of carriers of the homozygous polymorphic allele versus carriers of heterozygous and homozygous wild-type allele. For 1 selected SNP, a dominant model compared the homozygous wild-type carriers versus the heterozygous and homozygous SNP carriers. The models increased the statistical power and avoided the heterozygous effect observed in the linear model. Because this was an exploratory study, no adjustment for multiple testing was performed.

RESULTS

T_H17 lineage gene markers were expressed in CBMCs

Gene expression of T_H17 lineage markers was assessed in unstimulated and stimulated (PHA, LpA, and Ppg) cord blood samples. Expression of *RORC* (RORC3: isoform b; RORC4: isoforms a+b), *IL17*, *IL17F*, *IL23R*, and *IL22* was consistently increased after PHA, LpA, and Ppg stimulation ($P \leq .05$), whereas *RORA* expression was downregulated after stimulation ($P \leq .001$, Fig 1).

T_H17 cell markers were positively correlated with Treg cell markers at the mRNA level in cord blood

The correlation between markers of both cell populations after different stimulation conditions was assessed in cord blood to study putative reciprocal regulation of T_H17 and Treg cells early in life. mRNA expression of the T_H17 transcription factors *RORC* (RORC4: *RORC* isoform a+b) and *RORA* and *IL23R* were highly positively correlated with *FOXP3* mRNA after all stimulation conditions (PHA, LpA, and Ppg; Table II). Expression of isoform b of *RORC* (named *RORC3*) was highly positively correlated with *FOXP3* mRNA on PHA and Ppg stimulation, whereas expression of the *IL17*, *IL17F*, and *IL22* cytokines was positively correlated on innate (LpA and Ppg), but not PHA, stimulation (Table II). No significant correlations were detected in unstimulated conditions (not shown). Similar correlations as described for *FOXP3* were observed between T_H17 mRNA markers and other Treg cell–related markers, such as *LAG3* and *GITR* (data not shown).

T_H17 mRNA markers were not influenced by maternal farm exposure in cord blood

Farming is one of the strongest protective factors for the development of ADs.[33,34] We hypothesized that T_H17 cells, numbers of which are suggested to be increased in patients with ADs,[3-6] might be downregulated by farming exposure at birth.

IL23R mRNA expression was significantly lower in unstimulated cord blood of farming compared with nonfarming children ($P = .02$, Table III). No other significant differences in gene expression of T_H17 lineage markers were found between the 2 groups (Table III).

Because T_H17 and Treg cell mRNA markers were strongly correlated in cord blood (Table II), we analyzed the gene expression of T_H17 markers depending on maternal farm exposure, adjusting for *FOXP3* mRNA expression. An interaction effect was observed between Treg cells (*FOXP3* mRNA) and farming on the T_H17-related markers *IL22* ($P = .038$) and *IL23R* ($P = .052$) on LpA stimulation. This means that the relationship between T_H17-related markers and Treg cells differed significantly among farming and nonfarming mothers. No significant differences on T_H17 markers depending on farming status were observed (not shown). CBMCs from farm-exposed mothers (including farming mothers and mothers exposed to stables, barns, farm animals, and/or farm milk consumption) compared with non–farm-exposed mothers showed similar effects (data not shown).

Maternal farm exposure influenced T_H17 and Treg cell marker correlation on LpA stimulation

Because interaction effects were found in CBMCs between Treg cells (*FOXP3* mRNA) and maternal farming on T_H17 cell markers, we performed stratified analysis for farming and nonfarming mothers, respectively. We assessed the correlation coefficient of Treg cell *(FOXP3)* and T_H17 cell lineage markers separately in farming and nonfarming children (Table IV). A positive correlation was observed on stimulation (PHA, LpA, and Ppg) within CBMCs of nonfarming children (Table IV), whereas in the farming group gene expression of T_H17 and Treg cell markers was positively correlated on PHA and Ppg stimulation, although not in LpA-stimulated samples (Table IV).

Effect of RA on Treg and T_H17 cell markers in cord blood

RA, a derivate of vitamin A, is a key regulator of the T_H17/Treg cell balance.[17,35] Because our data suggest a correlation between T_H17 and Treg cell markers, we hypothesized that RA might influence T_H17/Treg cell regulation early in life. We analyzed

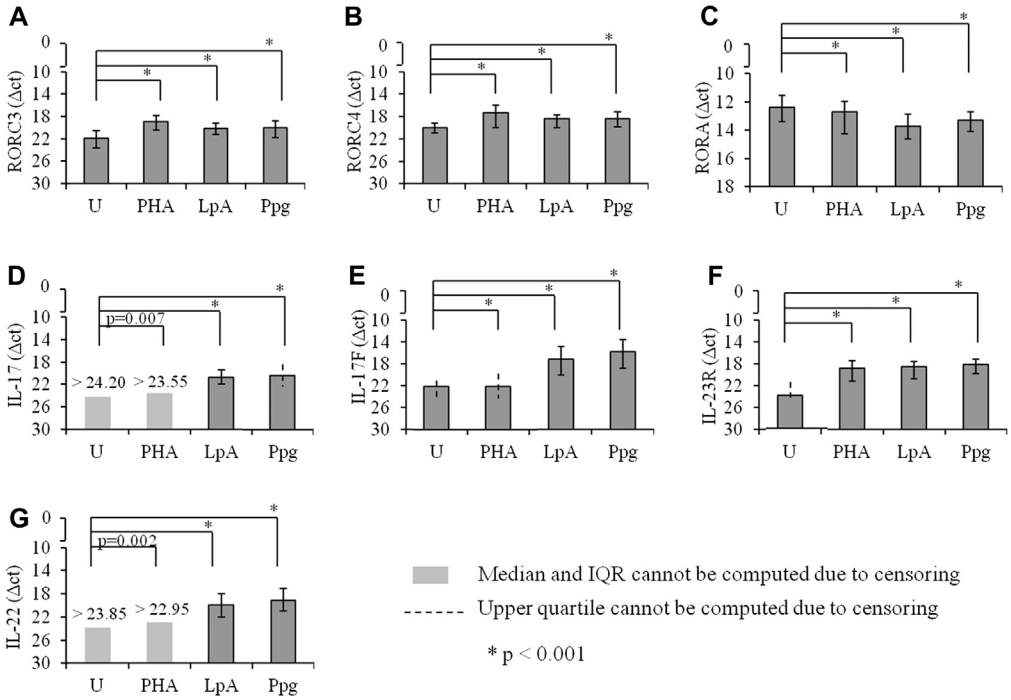

FIG 1. A-G, Gene expression of T_H17 lineage–related markers in CBMCs. ΔCT values are shown on the *y-axis*. As ΔCT values present low expression, the y-axis is shown *vice versa*. Lower ΔCT values between groups correspond to increased mRNA expression. Data are shown as the median ± interquartile range *(IQR)*. The paired Prentice-Wilcoxon test was used for analysis. *RORC3, RORC* isoform b; *RORC4, RORC* isoform a+b; *U*, unstimulated. *$P < .001$ (n = 67).

TABLE II. Correlation between *FOXP3* mRNA (Treg cells) and T_H17 lineage gene mRNA expression analyzed by using the Spearman correlation coefficient

Marker	Stimulus	No.	r*	P value
RORC3	PHA	54	**0.70**	**$<1 \times 10^{-5}$**
	LpA	44	0.26	.09
	Ppg	44	**0.41**	**.005**
RORC4	PHA	53	**0.70**	**$<1 \times 10^{-5}$**
	LpA	43	**0.55**	**2×10^{-4}**
	Ppg	43	**0.70**	**$<1 \times 10^{-5}$**
RORA	PHA	54	**0.55**	**2×10^{-5}**
	LpA	44	**0.58**	**4×10^{-5}**
	Ppg	44	**0.45**	**2×10^{-3}**
IL23R	PHA	54	**0.69**	**$<1 \times 10^{-5}$**
	LpA	44	**0.45**	**2×10^{-3}**
	Ppg	44	**0.37**	**.01**
IL17	PHA	54	0.07	.62
	LpA	44	**0.48**	**9×10^{-4}**
	Ppg	44	**0.77**	**$<1 \times 10^{-5}$**
IL17F	PHA	53	0.20	.16
	LpA	44	**0.54**	**1×10^{-4}**
	Ppg	44	**0.71**	**$<1 \times 10^{-5}$**
IL22	PHA	54	0.20	.15
	LpA	44	**0.47**	**1×10^{-3}**
	Ppg	44	**0.63**	**$<1 \times 10^{-5}$**

P values in boldface are significant.
RORC3, *RORC* isoform b; *RORC4*, *RORC* isoform a+b.
*Spearman correlation coefficient.

the effect of different doses of RA *in vitro* on unstimulated or PHA-stimulated CBMCs of healthy control children. Increasing doses of RA led to downregulation of Treg and T_H17 mRNA markers. *RORC* (namely RORC4 [*RORC* isoform a+b]) expression was downregulated on PHA stimulation with 50 and 100 nmol/L RA, whereas *IL23R* was downregulated in both unstimulated and PHA-stimulated cells after all doses of RA ($P \leq .05$, Fig 2). *FOXP3* expression was downregulated in both unstimulated and PHA-stimulated cells after 50 and 100 nmol/L RA ($P < .05$, Fig 2).

SNPs within the T_H17 lineage–related genes influenced both T_H17 and Treg cell markers in cord blood

To assess potential effects of polymorphisms on complex immune regulation, we studied the effect of SNPs within the T_H17 lineage genes on T_H17 and Treg cell markers. Seven SNPs located within the *IL17*,[25] *IL23R*, and *RORC* genes, which have been shown by Schieck et al[36] to be relevant for childhood asthma or to have a putative role in IL-17 regulation,[24] were genotyped (Table I).

Applying a recessive model, homozygous SNP carriers of *IL23R* rs7517847 showed downregulation of T_H17 lineage markers for *IL23R* (LpA and Ppg), *IL17* (LpA), *IL17F* (unstimulated, LpA, and Ppg), and *IL22* (LpA) mRNA expression and IL-17 (LpA and Ppg) protein secretion ($P \leq .04$, Table V and see Table E2 in this article's Online Repository at www.jacionline.org). A similar trend was observed for homozygous carriers of the *IL17* SNP rs2275913. The *IL23R* SNP rs790631 revealed an upregulation of T_H17 lineage markers for the homozygous SNP carriers in *RORC* (isoform b; Ppg) and *IL17F* (unstimulated) gene expression but a downregulation of *IL23R* (PHA) and *FOXP3* (Ppg) mRNA ($P \leq .05$, Table V and see

TABLE III. Gene expression of T_H17 markers in CBMCs from neonates of farming mothers compared with nonfarming mothers (n = 67)

Stimulus	Marker	Estimate	*P* value	Stimulus	Marker	Estimate	*P* value
U	*RORC3*	12.04	.08	LpA	*RORC3*	6.45	.21
	RORC4	−3.94	.51		*RORC4*	−0.59	.92
	RORA	−1.42	.80		*RORA*	−2.65	.60
	IL23R	**23.54**	**.02**		*IL23R*	1.70	.76
	IL17	448.72	1.00		*IL17*	−0.24	.97
	IL17F	−9.85	.27		*IL17F*	−2.81	.66
	IL22	17.50	.39		*IL22*	−3.98	.48
PHA	*RORC3*	0.36	.95	Ppg	*RORC3*	1.13	.83
	RORC4	−4.82	.39		*RORC4*	1.22	.82
	RORA	−3.25	.54		*RORA*	3.30	.51
	IL23R	−8.88	.15		*IL23R*	−2.99	.59
	IL17	5.23	.74		*IL17*	−1.20	.87
	IL17F	−12.98	.11		*IL17F*	−2.70	.62
	IL22	−13.22	.17		*IL22*	−3.08	.61

A positive estimate resembles lower gene expression within the farming group. Analysis was done with Tobit regression on rank-transformed data. *P* values in boldface are significant.
RORC3, *RORC* isoform b; *RORC4*, *RORC* isoform a+b; *U*, unstimulated.

TABLE IV. Correlation between *FOXP3* mRNA (Treg cells) and T_H17 lineage gene mRNA expression in CBMCs stratified by maternal farming status

		Farmers			Nonfarmers		
Stimulus	Marker	No.	*r*	*P* value	No.	*r*	*P* value
U	*RORC3*	17	0.17	.51	41	0.05	.74
	RORC4	17	0.43	.09	40	0.24	.14
	RORA	17	0.28	.27	41	**0.44**	**.004**
	IL23R	17	0.25	.33	41	**0.56**	**2×10^{-4}**
	IL17	17	0.16	.54	41	0.13	.41
	IL17F	17	0.004	.99	41	0.05	.75
	IL22	17	0.34	.18	41	0.14	.37
PHA	*RORC3*	17	**0.62**	**.01**	37	**0.70**	**$<1 \times 10^{-5}$**
	RORC4	17	**0.79**	**2×10^{-4}**	36	**0.65**	**2×10^{-5}**
	RORA	17	**0.65**	**.005**	37	**0.50**	**.002**
	IL23R	17	**0.66**	**.004**	37	**0.73**	**$<1 \times 10^{-5}$**
	IL17	17	−0.09	.74	37	0.14	.42
	IL17F	17	0.17	.52	36	0.22	.20
	IL22	17	0.11	.67	37	0.26	.12
LpA	*RORC3*	12	0.52	.08	32	0.19	.30
	RORC4	12	0.56	.06	31	**0.54**	**.002**
	RORA	12	0.35	.27	32	**0.66**	**3×10^{-5}**
	IL23R	12	0.20	.53	32	**0.68**	**2×10^{-5}**
	IL17	12	0.32	.31	32	**0.57**	**7×10^{-4}**
	IL17F	12	0.38	.22	32	**0.64**	**7×10^{-5}**
	IL22	12	0.20	.53	32	**0.63**	**1×10^{-4}**
Ppg	*RORC3*	12	**0.65**	**.02**	32	0.30	.10
	RORC4	12	**0.86**	**4×10^{-4}**	31	**0.60**	**4×10^{-4}**
	RORA	12	0.26	.42	32	**0.47**	**.01**
	IL23R	12	0.34	.27	32	0.31	.08
	IL17	12	**0.77**	**.003**	32	**0.73**	**$<1 \times 10^{-5}$**
	IL17F	12	**0.62**	**.03**	32	**0.67**	**2×10^{-5}**
	IL22	12	**0.66**	**.02**	32	**0.61**	**2×10^{-4}**

P values in boldface are significant ($P \leq .05$).
RORC3, *RORC* isoform b; *RORC4*, *RORC* isoform a+b; *U*, unstimulated.

Table E2). In our population only 1 homozygous SNP carrier was observed for the *IL23R* SNP rs10889675, and thus a dominant model was applied. Downregulation of T_H17 lineage markers (unstimulated: *RORC* isoform b, *RORA*, and *IL22*) and upregulation of *FOXP3* (LpA) were found at the mRNA level. At the protein level, IL-5 (PHA and Ppg), IL-13 (PHA), IL-6 (PHA), and IL-15 (PHA and LpA) were downregulated ($P \leq .05$, Table V and see Table E2). No significant effects were observed for the other SNPs or in the studied cytokines (data not shown).

DISCUSSION

This study investigated underlying immunologic mechanisms of "atopy protection through farm exposure" early in life by assessing regulation of T_H17 cell markers. T_H17 cells are already expressed in cord blood independent of maternal farming. Their close regulation with Treg cells is dependent on maternal farming status. Specifically, T_H17 lineage mRNA expression was highly positively correlated with *FOXP3* mRNA expression for both farming and nonfarming children after adaptive (PHA) and innate Ppg stimulation. For nonfarming children only, a positive correlation was also present after LpA stimulation. Regarding genetic regulation, SNPs within the T_H17 lineage genes influenced gene expression of T_H17 and Treg cell markers and T-cell cytokine secretion.

Several studies have now replicated that early-life farm exposure is protective for the development of ADs,[10-12,33,34] particularly when exposure occurs prenatally.[15] Although we have previously shown that Treg cell numbers were increased and functionally more efficient in the cord blood of farming children, they are described to be regulated in concert with other T-cell populations, such as T_H17 cells. For example, Treg cells are known to limit the immunopathology of T_H17-driven inflammatory bowel disease,[37] a higher ratio of T_H17/Treg cells has been related to graft-versus-host disease,[38] and murine studies have demonstrated that $FOXP3^+$ Treg cells are required for the development of T_H17 cells *in vivo* through IL-2 regulation.[39]

In this context 3 main findings of our study are important, comprising the effect of farming, the correlation of Treg and T_H17 cell markers, and genetic influences. First, prenatal farming exposure had no influence on T_H17 lineage mRNA expression in cord blood *per se*; only *IL23R* was differently expressed at baseline. These data are in support of our previous results in the same birth cohort, in which no differences in IL-17 protein secretion were shown between farming and nonfarming CBMCs. Of note, IL-5 secretion was lower in children with pregnancy farm exposure on allergen and innate stimulation in our previous study.[16]

Second, the high positive correlation of T_H17-related mRNA expression with *FOXP3* mRNA expression, the transcription factor of Treg cells, was also independent of farming after PHA and Ppg stimulation. The positive correlation between *FOXP3* and *RORA* or *IL23R* mRNA expression, respectively, at baseline was only observed in nonfarming children. Potentially, prenatal exposures other than farming might influence their mRNA expression. Interestingly, after stimulation with LpA, the main component of endotoxin, the T_H17/Treg cell correlation was only present in nonfarming children, potentially suggesting a stimulus-specific T_H17/Treg mRNA correlated effect in children with low exposure or, alternatively, no effect in previously exposed children. This is consistent with the interaction effect observed between Treg cells and farming on T_H17 lineage genes on LpA stimulation in this study. The overall positive correlation

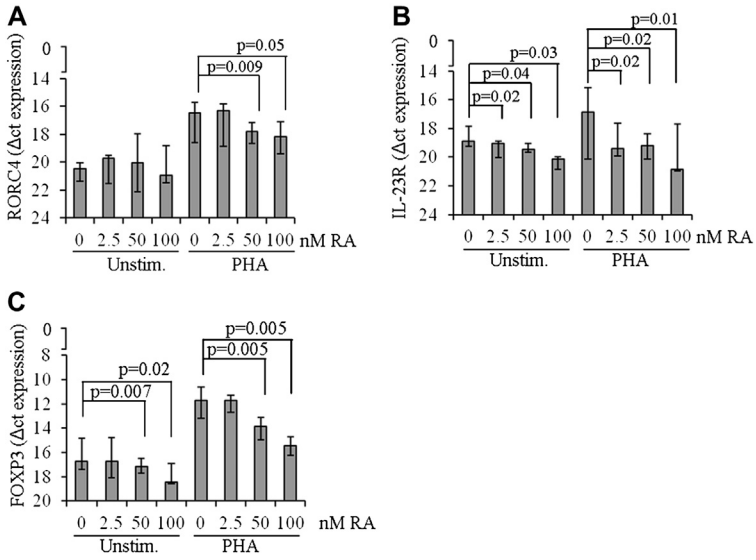

FIG 2. A-C, Effect of RA on T_H17 and Treg cell markers in CBMCs. ΔCT values are shown on the *y-axis*. As ΔCT values present low expression, the y-axis is shown *vice versa*. Lower ΔCT values between groups correspond to increased mRNA expression. Data are shown as the median ± interquartile range *(IQR)*. The paired Prentice-Wilcoxon test was used for analysis (n = 7). *RORC4, RORC* isoform a+b.

TABLE V. Summary of gene expression of T_H17/Treg cell markers and cytokine secretion for each analyzed SNP

Recessive model

		T_H17														Treg
		mRNA												Protein		mRNA
Gene	rs no.	*RORC*		*RORA*		*IL23R*		*IL17*		*IL17F*		*IL22*		IL-17		*FOXP3*
IL23R	rs7517847 GG	ND	↓	LpA	↓	LpA* Ppg*	↓	LpA*	↓	U* LpA* Ppg*	↓	LpA*	↓	LpA* Ppg*		ND
	rs790631 CC	↑ Ppg*		ND	↓	PHA*		ND	↑	U*		ND		ND	↓	PHA Ppg*
IL17	rs2275913 AA	ND	↓	LpA Ppg	↓	PHA	↓	LpA	↓	Ppg	↓	Ppg		ND		ND

Dominant model

		T_H17														Treg
		mRNA												Protein		mRNA
Gene	rs no.	*RORC*		*RORA*		*IL23R*		*IL17*		*IL17F*		*IL22*		IL-17		*FOXP3*
IL23R	rs10889675 AA	↓ U*		↓ U*		ND		ND	↓	U	↓	U*	↓	Ppg	↑	LpA* Ppg

		Protein													
Gene	rs no.	IFN-γ		IL-5		IL-13		IL-6		IL-15		TNF-α		GM-CSF	
IL23R	rs10889675 AA	ND	↓	PHA* LpA Ppg*	↓	PHA*	↓	PHA*	↓	PHA* LpA*	↓	PHA	↓	LpA	

↑, Expression upregulated; ↓, expression downregulated; *ND*, no difference; *RORC3*, *RORC* isoform b; *U*, unstimulated.
*$P \leq .05$.

between T_H17-related and *FOXP3* mRNA expression indicates a parallel regulation at this early stage of immune development. In parallel, recent findings from a murine study showed that Treg cells promoted T_H17 cell differentiation.[39] This parallel regulation was only observed in early phases of differentiation, and inhibition of T_H17 cell responses by Treg cells might occur during later phases of inflammation.[39] Thus the positive correlation in our study might be relevant during early T_H17 cell differentiation, and in cord blood both subsets might still be regulated in parallel, subsequently affecting further lineage fate depending on external influences, including the cytokine milieu[40] and the presence of regulatory metabolites.[17] Furthermore, the fate of both subsets might be genetically regulated through interaction between their transcription factors, such as through

direct interaction or involvement of other transcription factors, such as Runt-related transcription factor 1.[41,42] Indeed, a certain plasticity has been observed on Treg cells that can develop a T_H17-like phenotype *in vivo*.[43] Of note, the assessed T_H17 lineage markers in our study are characteristic for but not exclusively expressed in T_H17 cells[44] and were assessed based on mRNA expression. Additionally, this study was performed in unfractionated CBMCs, and thus the cell source cannot be exactly specified; however, a consistent expression of several genes points to a common pattern.

Regarding influences on T-cell fate early in life, in this study RA-treated cord blood samples showed a downregulation of T_H17 (*RORC* and *IL23R*) and Treg *(FOXP3)* cell markers with a dose-response effect occurring particularly on PHA stimulation. RA, a vitamin A derivate, is relevant for immune cell differentiation and maintenance of immune homeostasis[35] and can enhance induced Treg cells[17,45] while suppressing T_H17 differentiation in mice.[17] In human subjects *in vitro* treatment with RA increased FOXP3 expression and decreased IL-17 expression in colon biopsy specimens from patients with ulcerative colitis.[35] Thus during inflammation, it seems to be a key regulator of these 2 T-cell subsets in opposite directions. The parallel regulation of both T_H17 and Treg cell markers in CBMCs after RA treatment in our study is in concordance with the overall regulatory pattern. Our findings after RA stimulation might either indicate a specific, parallel, strong T_H17/Treg cell regulation early in life or a dose-dependent response to RA (although doses are comparable with other studies) or might simply require additional functional investigations for T_H17/Treg cell regulation. In this study a more detailed assessment of T_H17 and Treg cells by using flow cytometry and additional functional studies were not feasible because of logistics and limited cell numbers. Finally, genetic influences of T_H17-associated markers on T-cell regulation were already present early in life. The influence of the polymorphism in *IL23R* rs7517847 on T_H17 cell marker expression is in line with findings from a genome-wide association study, in which the SNP was protective for inflammatory bowel disease, a potentially T_H17-driven disease.[46] In contrast to our results, a Chinese study on school-age children assessing the *IL17* polymorphism rs2275913 did not show any difference in IL-17 secretion. However, carriers of the SNP were more likely to have childhood asthma,[25] which has now also been shown for children with nonatopic asthma in a study from Schieck et al (submitted manuscript). In our study homozygous carriers of the *IL23R* SNP rs790631 showed increased T_H17 marker and decreased *FOXP3* expression, whereas carriers of the *IL23R* SNP rs10889675 had decreased T_H17 and T_H2 cell marker expression and increased *FOXP3* mRNA expression. Although generally Treg/T_H17 mRNA expression was regulated in parallel in our birth cohort, also after RA treatment, genetic polymorphisms influenced mRNA expression in opposite ways, potentially being strong regulators. Indeed, previous studies demonstrated the relevance of specific T_H cell–related SNPs on immune regulation closely related to ADs.[26,47]

Although the size of this cohort is smaller compared with that in other farming studies, the strength of this analysis is the comprehensive immunologic evaluation of study subjects very early in life, when the immune system is susceptible to the development of ADs influenced by both environmental and genetic factors. Replication of our data in further larger studies will contribute to disentangle these important regulatory immune mechanisms after environmental exposure for the development of childhood ADs.

In summary, our study proposes a parallel regulation of T_H17 and Treg cell markers early in life not influenced by "atopy-protective" farming exposure *in vivo* or by RA treatment *in vitro*. However, a T_H17/Treg cell correlation was not observed on LpA stimulation in cord blood from farming neonates, suggesting a potential role for prenatal farming exposure on the interaction between these 2 populations. Furthermore, T_H17 polymorphisms influenced T_H17/Treg cell marker expression, indicating an influence of genetic predisposition on T-cell regulation. These findings might be relevant for early immune differentiation in the context of an "atopy-protective environment." However, only a detailed follow-up including functional studies on T-cell and other cell populations of this and additional cohorts will elucidate whether these effects can have an effect on the development of ADs in childhood.

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Key messages

- **Maternal farm exposure did not affect T_H17 lineage markers in cord blood but influenced T_H17 and Treg cell marker correlation.**
- **SNPs within the T_H17 pathway influenced gene expression of T-cell markers and cytokine secretion in CBMCs.**

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