

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

Bianca Schaub, MD,^a Jing Liu, MD,^{a,c} Sabine H  ppler,^a Severine Haug,^a Christine Sattler, MD,^b Anna Llu  s,^a Sabina Illi, PhD,^a and Erika von Mutius, MD, MSc^a Munich, Germany, and Chang Chun, China

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

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

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

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

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

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

Abbreviations used

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

Maternal atopy is a critical risk factor for the development of childhood allergic diseases¹ potentially by modulating the child's immune system. However, underlying immunological mechanisms are not well determined. Epidemiologic studies suggest that immune modulation and potential protection against allergies may occur early in life.² Maternal atopy may be one important factor interacting with environmental stimuli influencing a child's immune system at an early time point, perhaps even *in utero*.³

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

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

From ^athe Department of Pulmonary and Allergy, University Children's Hospital Munich, and ^bthe Department of Gynecology, University Hospital, Ludwig-Maximilians-University, Munich; and ^cthe Second Hospital of JI LIN University, Department of Respiratory Medicine, Chang Chun.

Supported by Deutsche Forschungs Gemeinschaft 997/2-1, Friedrich Baur Institute, FoFoLe, and Stiftung Omnibus (all B.S.).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication October 31, 2007; revised April 4, 2008; accepted for publication April 14, 2008.

Reprint requests: Bianca Schaub, MD, University Childrens Hospital Munich, Dr von Haunersches Kinderspital, Lindwurmstr 4, 80337 Munich, Germany. E-mail: Bianca.Schaub@med.uni-muenchen.de.

0091-6749/\$34.00

   2008 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2008.04.010

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

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

METHODS

For details please see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Study population

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

Lymphocyte proliferation, cytokine secretion of CBMCs

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

Real-time quantitative RT-PCR

Total RNA, isolated with TRI Reagent (Sigma, Taufkirchen, Germany) was processed with reverse transcriptase (Invitrogen, Karlsruhe, Germany). mRNA-specific oligonucleotide primer pairs were designed with Vector NTI-Advance10 (Invitrogen) (see the [Methods](#) section in the Online Repository at www.jacionline.org). Direct detection of the PCR product was monitored by measuring the increase in fluorescence caused by binding of SYBR-Green (Applied Biosystems, Darmstadt, Germany) to double-stranded DNA (dsDNA). The determined threshold cycle (CT) was set relative to the

amplification plot of 18S ribosomal RNA (18SrRNA). The CT is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold set to the log-linear range of the amplification curve. The difference in CT values relative to the housekeeping gene was used to calculate the fold difference. The relative quantitative results were used to determine changes of stimulated/unstimulated samples.¹⁶

Flow cytometry, functional analysis of Treg/T_H17 cells

Cells were analyzed by using 3-color flow cytometry (FACScan; Becton-Dickinson, Heidelberg, Germany). For surface staining, 2 μ L antihuman CD4-fluorescein isothiocyanate (FITC), CD25-R-phycoerythrin-Cyanin 5 (RPE-Cy5), 1 μ L IgG₁-FITC (Dako Cytomation, Glostrup, Denmark), and 0.5 μ L IgG_{2a}-RPE-Cy5 (BD Biosciences, Heidelberg, Germany; Pharmingen, Hamburg, Germany) were used. For intracellular Foxp3/IL-17 staining, 8 μ L antihuman CD4-FITC and 4 μ L antihuman CD25-RPE-Cy5 antibodies (1×10^6 /100 mL) were used, cell permeabilization was performed, and Foxp3-phycoerythrin/antihuman IL-17 antibody/corresponding isotype control was added. Data were analyzed with CellQuest software (Becton-Dickinson), and postacquisition analysis was performed with WinMDI 2.8 software (Becton-Dickinson). Regarding functional Treg cell studies, CD3⁺ cells were isolated (CD3 isolation kit; Miltenyi Biotec, Köln, Germany) and irradiated. CD4⁺CD25⁻/CD4⁺CD25⁺ T cells were isolated (2-step procedure) by using depletion of non-CD4⁺ cells, followed by positive selection of CD4⁺CD25⁺ T cells (Miltenyi Biotec). CD4⁺CD25⁻ T cells (2×10^4 /well), labeled with 5 μ mol/L carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen), were incubated for 3 days with irradiated CD3⁺ cells (4×10^4 /well) in coculture with/without CD4⁺CD25⁺ T cells (2×10^4 /well) before/after stimulation with 0.8 μ g/mL phytohemagglutinin. Division and proliferation of CD4⁺CD25⁻ T cells were assessed; cytokine concentrations were measured in supernatants by using the Human Cytokine-Multiplex-Assay-Kit.

Statistical analysis

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

RESULTS

Population characteristics

Of all healthy mothers (n = 161), complete epidemiologic data and sufficient cord blood for detailed immunological analysis were available from 118 mothers/neonates (73%) complying with the inclusion criteria ([Table I](#); [Methods](#) in the Online Repository). Of 118 mothers, 48 mothers were affected by asthma and/or allergic rhinitis and/or atopic eczema. Eighty-five percent of these mothers also had a positive RAST test. The reference group did not have any atopic disease and had sensitization levels comparable to previous epidemiologic studies among nondiseased subjects¹⁷ ($P < .001$). The 2 groups of mothers with and without atopy differed further significantly regarding neonatal birth characteristics ($P \leq .02$)

TABLE I. Population characteristics in CBMC of 2 groups of mothers with and without atopy

	No maternal atopy (n = 70)	Maternal atopy* (n = 48)	P value (χ^2 test or Wilcoxon)
Maternal age (y)‡	33 (31/36)	32.5 (31/36)	.81
Smoking during pregnancy, n (%)			
No smoking	59 (84.3)	44 (91.7)	.24
During pregnancy	4 (5.7)	3 (6.3)	.79
Until pregnancy confirmed	7 (10)	1 (2.1)	
Median no. of cigarettes	0 (0/20)	0 (0/10)	
Maternal total serum IgE (IU/mL)‡	21.18	60.54	<.0001
Maternal specific IgE >0.7 IU/mL (%)	43.5	85.4	<.001
Male sex, n (%)	34 (48.6)	36 (51.4)	.30
Gestational age (wk)‡	40 (39.1/40.7)	39.9 (38.9/40.9)	.99
Birth weight (g)‡	3450 (3150/3700)	3695 (3485/3937)	.008
Birth length (cm)‡	52 (50/53)	53 (51/54)	.02
Head circumference (cm)‡	35.0 (35/36)	35.4 (35/36.75)	.02
Neonatal race/ethnicity, n (%)			
European	68 (97.1)	46 (95.8)	.69
Non-European	2 (2.9)	2 (4.2)	
Vaginal delivery, n (%)	64 (91.4)	44 (91.7)	.91
Apgar 5 minutes >8 (%)	100	100	1.0
Older siblings, n (%)			.35
0	44 (62.9)	27 (56.3)	
1	23 (32.9)	15 (31.3)	
2	3 (4.3)	4 (10.4)	
3	0 (0)	1 (2.1)	
Miscarriage, n (%)			.52
N = 1	13 (18.6)	12 (25)	
N = 2	1 (1.4)	0 (0)	
Maternal education, n (%)			.90
Elementary school	3 (4.3)	3 (6.2)	
Middle school	10 (14.3)	7 (14.5)	
High school	10 (14.3)	5 (10.4)	
University	47 (67.1)	33 (68.8)	
Previous cesarian section, n (%)	3 (4.3)	3 (6.3)	.63
Paternal atopy, n (%)*	17 (24.3)	21 (43.75)	.03
Asthma	3 (4.3)	5 (10.4)	.27†
Hay fever	16 (22.9)	15 (31.3)	.31
Atopic eczema	2 (2.9)	6 (12.5)	.06†

Data in **boldface** are significant.

*Maternal or paternal atopy was defined as doctor's diagnosis of asthma, allergic rhinitis, and/or atopic eczema.

†Fisher's exact test.

‡For median $\pm$ IQR (interquartile range).

and paternal atopy ($P = .03$). There were no differences regarding delivery mode, previous cesarean section, or older siblings.

Lower glucocorticoid-induced TNFR-related protein and lymphocyte activation gene 3 but not Foxp3 expression in CBMCs with maternal atopy

We determined mRNA-expression of Treg cell-associated genes, namely, the most specific one, Foxp3, and glucocorticoid-induced TNFR-related protein (GITR), lymphocyte activation gene 3 (LAG3), in addition to cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and TGF- β . LpA-stimulated and peptidoglycan-stimulated GITR and LAG3 expression of offspring from nonatopic compared with atopic mothers was significantly higher or borderline significant (Fig 1, A and B). Foxp3, CTLA4, and TGF- β expression were higher but not significant in LpA/peptidoglycan-stimulated CBMCs (Fig 1; for TGF- β , $P = .72/.62$). Baseline values were not significantly different. Paternal atopy had no significant influence on gene expression apart from a minor change in LAG3 (see the Results section of the Online Repository at www.jacionline.org).

Correlation of Treg cell-associated genes for all subjects revealed significant positive correlations of GITR/LAG3 for all stimulations besides peptidoglycan (see this article's Table E1 in the Online Repository at www.jacionline.org). Maternal atopy was important in LpA-stimulated and D+L-stimulated CBMCs. In contrast, no atopy influenced the correlation in phytohemagglutinin-stimulated and *D pteronyssinus* 1-stimulated cells. Atopy had no (*D pteronyssinus* 1) or minor effects (phytohemagglutinin) on the correlation. Correlations of GITR/Foxp3 or LAG3/Foxp3, respectively, showed primarily significant increases in both for phytohemagglutinin-stimulated and D+L-stimulated no atopy. This may point to differences in gene expression depending on the maternal atopic phenotype in association with innate or adaptive/mitogenic immune stimuli. For paternal atopy, no significant changes were observed (not shown).

CD4⁺CD25⁺high T cells were significantly lower in CBMC with maternal atopy

Assessing CD4⁺CD25⁺ T cells, composed of activated T cells and Treg cells, there were no significant differences apart

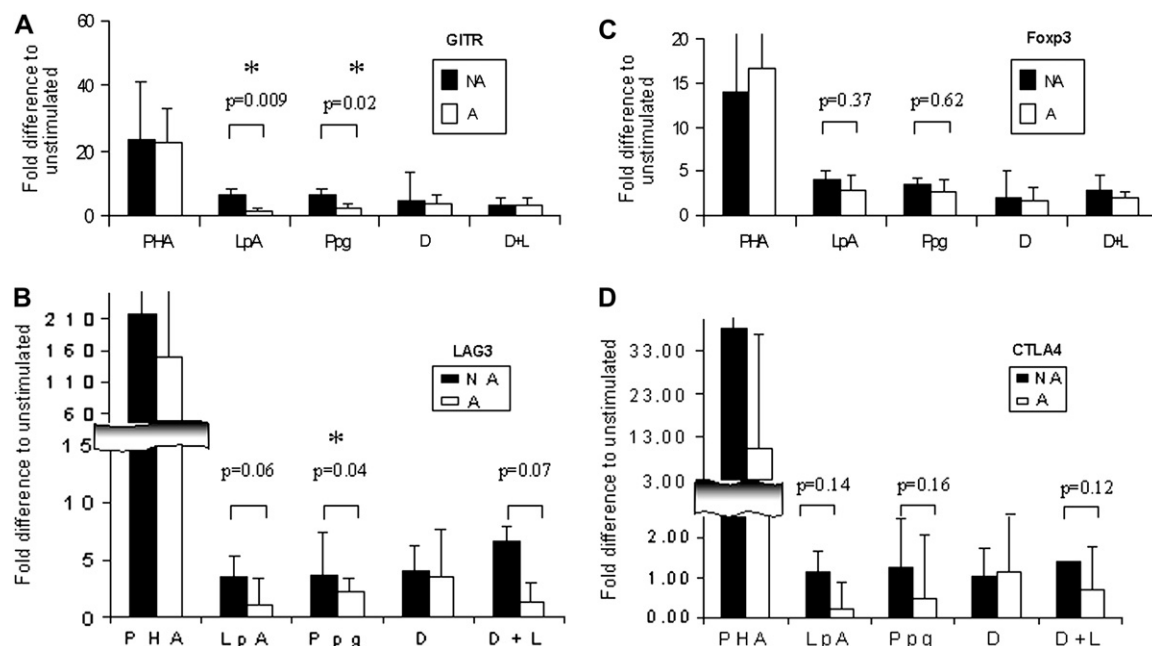


FIG 1. The level of mRNA expression was shown as fold difference in gene expression in phytohemagglutinin (PHA; 5 μ g/mL), LpA (0.1 μ g/mL), peptidoglycan (Ppg; 10 μ g/mL), *D pteronyssinus* 1 (D; 30 μ g/mL), and D+L stimulated/unstimulated samples, compared with the housekeeping gene 18S. RNA was prepared as described in Methods. **A-D**, Quantitative gene expression was assessed using real-time RT-PCR. NA, Cord blood of nonatopic mothers; A, cord blood of atopic mothers.

from unstimulated CD4⁺CD25⁺ T cells (%) depending on maternal atopy (Table II). Analyzing data in a dichotomized way (below/above median) and calculating ratios to unstimulated values did not change results significantly (not shown). Analyzing CD4⁺CD25⁺high T cells (total and ratio/unstimulated), most closely representing Treg cells, we detected significantly lower CD4⁺CD25⁺high T cells in LpA/peptidoglycan-stimulated CBMCs with maternal atopy ($P = .005/.05$), respectively (Table II/see this article's Fig E1 in the Online Repository at www.jacionline.org). Foxp3 staining of CD4⁺CD25⁺high cells was positive in 70.9% (mean $\pm$ 11.2 SEM) for unstimulated, 79.9% ($\pm$ 3.3) for phytohemagglutinin-stimulated, 86.6% ($\pm$ 1.4) for LpA-stimulated, and 92.2% ($\pm$ 0.8) for peptidoglycan-stimulated cells. For CD4/CD8/CD19, no significant differences depending on maternal atopy were observed (not shown).

IFN- γ and IL-10 secretion were significantly decreased in LpA-stimulated CBMCs of mothers with atopy

Lymphocyte proliferation and cytokine secretion by Treg cells and T_H1/T_H2 subpopulations was overall not significantly different after stimulation depending on maternal atopy (Table II; see this article's Table E2 in the Online Repository at www.jacionline.org; see Methods in the Online Repository). However, LpA-stimulated IFN- γ and IL-10 were lower in CBMCs with maternal atopy similarly after subtraction of unstimulated values (see this article's Table E3 in the Online Repository at www.jacionline.org). *D pteronyssinus* 1-stimulated and D+L-stimulated IL-10 and IFN- γ showed trends to lower values in CBMCs with maternal atopy. When analyzing the few normally distributed cytokines with *t* test or after log-transformation, *D pteronyssinus* 1-stimulated IL-10 showed a trend to significance ($P = .06$). In logistic

regression analysis, IL-10 secretion was lower in stimulated CBMCs (phytohemagglutinin, LpA, *D pteronyssinus* 1, D+L) of atopic compared with nonatopic mothers, although only significant for LpA and borderline significant for D+L. *D pteronyssinus* 1-induced IFN- γ showed a lower OR of 0.43 (95% CI, 0.19-0.95; $P = .03$) with maternal atopy. Furthermore, phytohemagglutinin-induced proliferation showed an OR of 2.29 (95% CI, 1.03-5.06; $P = .04$) for maternal atopy.

Paternal atopy and birth characteristics had no influence on cytokine secretion and CD4⁺CD25⁺high expression

Paternal atopy showed a significant association with maternal atopy. However, paternal atopy was not associated with any of the outcome variables, including CD4⁺CD25⁺high expression.

Furthermore, birth characteristics showed a significant association with maternal atopy. Birth characteristics revealed significant, though low ($r < 0.4$), correlations with some outcome variables (eg, IFN- γ -*D pteronyssinus* 1, IL-13-phytohemagglutinin/LpA/peptidoglycan/D+L). However, when included in the logistic regression analysis in addition to maternal atopy, the ORs for maternal atopy remained basically unchanged (not shown).

Correlations of cytokine secretions and Treg cell-associated genes

Assessing correlations of IL-10, secreted by Treg and T_H2 cells, with T_H2/T_H1 cytokines, significant correlations were present for IL-10 with both T_H2/T_H1 and TNF- α secretion, although with low correlation coefficients (see this article's Table E4 in the Online Repository at www.jacionline.org). IFN- γ , IL-13, and TNF- α were positively correlated with each other. Assessment for correlations

TABLE II. Two groups of CBMCs with and without maternal atopy: surface expression of CD4⁺CD25⁺ cells (%), lymphocyte proliferation (LP), and cytokine secretion (pg/mL) after stimulation with mitogen phytohemagglutinin (PHA), the innate stimuli LpA, peptidoglycan (Ppg), or allergen *D pteronyssinus* 1 (D)

Parameter	Stimulus	NA (mean ± SEM or median ± IQR)	A (mean ± SEM or median ± IQR)	P value (comparison of NA-A)
CD4 ⁺ CD25 ⁺ (%)	U	2.68 (0.24)	1.74 (0.32)	.03†
	PHA	26.81 (5.46)*	37.28 (7.51)*	.29
	LpA	1.84 (0.32)	1.75 (0.55)	.89
	Ppg	2.70 (0.64)	2.15 (0.61)	.55
CD4 ⁺ CD25 ⁺ high (%)	U	0.86 (0.30)	0.51 (0.14)	.36
	PHA	13.30 (3.43)*	17.87 (3.90)*	.41
	LpA	1.35 (0.55)	0.13 (0.21)	.005†
	Ppg	2.09 (0.38)	0.67 (0.38)	.05†
LP (SI)	PHA	16.1 (9.7/23.8)**	19.26 (12.37/37.27)**	.08
	LpA	1.03 (0.78/1.45)	1.09 (0.86/1.34)*	.76
	Ppg	1.45 (1.04/2.13)**	1.46 (1.04/2.49)**	.82
	D	1.28 (0.88/1.76)**	1.32 (0.96/1.88)**	.79
	D+L	1.10 (0.85/1.59)	1.25 (0.90/2.08)*	.32
IFN-γ	U	0.01 (0.01/0.74)	0.01 (0.01/ 1.99)	.61
	PHA	132.20 (20.50/261.64)**	95.73 (13.34/160.34)**	.30
	LpA	27.74 (5.35/128.42)**	12.38 (3.32/69.33)**	.04†
	Ppg	28.17 (9.26/137.75)**	24.16 (6.51/80.68)**	.14
	D	50.34 (7.03/160.00)**	18.41 (6.28/118.62)**	.11
IL-10	D+L	137.31 (16.28/167.32)**	63.42 (9.06/151.32)**	.21
	U	0.16 (0.12/0.24)	0.15 (0.10/0.21)	.18
	PHA	145.06 (69.30/234.86)**	103.14 (55.35/215.85)**	.27
	LpA	197.88 (130.58/337.15)**	167.01 (101.93/236.37)**	.05†
	Ppg	753.24 (171.75/1445.91)**	550.32 (49.95/1222.04)**	.40
	D	204.13 (110.78/397.23)**	178.83 (90.58/263.40)**	.10
	D+L	302.33 (205.62/477.92)**	228.06 (153.40/352.99)**	.07

A, CBMCs from offspring of atopic mothers; NA, CBMCs from offspring of nonatopic mothers.

Data in **boldface** are significant.**P* < .05, significant differences to unstimulated values per group.†Comparison between CBMCs of nonatopic and atopic mothers with *t* test (CD4⁺CD25⁺) or Mann-Whitney rank test (LP, cytokines).***P* < .001, highly significant differences to unstimulated values per group.

with both or no atopic parents revealed significant correlations for T_H1/T_H2 cytokines. Thus, parental atopy seems to change correlations of T_H1/T_H2 cytokines after different innate conditions.

Correlating IL-10 with Treg cell-associated genes, IL-10 increased significantly only with phytohemagglutinin-induced GITR and LAG3; however, correlations were low (see this article's [Table E5](#) in the Online Repository at www.jacionline.org). IL-10 and Foxp3 mRNA expression were not correlated. In addition, we assessed Foxp3 protein in CD4⁺CD25⁺ cells, showing a mean expression of 1.5% (±0.7 SEM) for unstimulated, 4.6% (±2.5) for phytohemagglutinin-stimulated, 2.2% (±0.5) for LpA-stimulated, and 1.2% (±0.7) for peptidoglycan-stimulated cells. This in addition to IL-10 secretion by T_H2 cells may contribute to the missing correlation between Foxp3 and IL-10 in CBMCs.

Functional differences in Treg cells depending on maternal atopy

We further assessed functional differences of Treg cells depending on maternal atopy. Mitogen-stimulated T-cell division was significantly increased in CD4⁺CD25⁺ T effector cells, consistent with increased proliferation (SI-phytohemagglutinin) in CBMCs ([Table II](#)). After addition of CD4⁺CD25⁺ T cells, effector cell division was significantly suppressed, and in a dose-dependent manner (80%, 65.5%, 19.6%; one representative example in this article's [Fig E2, D-F](#), in the Online Repository at www.jacionline.org). Comparing suppressive capacity of Treg

cells for T-cell division and proliferation, Treg cells from offspring without maternal atopy suppressed proliferation more efficiently than Treg cells from offspring with maternal atopy ([Fig 2, A and B](#)). This effect was again dose-dependent.

Assessing a potential change in the balance of T_H2/T_H1 cytokines, IL-10 and GM-CSF were suppressed in effector cells of nonatopic mothers, but not significantly. IFN-γ and TNF-α secretion were significantly suppressed in a dose-dependent manner. In atopic mothers, no significant cytokine suppression was observed. Comparing suppression of effector cells with and without maternal atopy, there was a trend to more suppression of IFN-γ and TNF-α in cord blood of nonatopic mothers ([Fig 3, C-F](#); see this article's [Fig E3](#) in the Online Repository at www.jacionline.org).

Role of T_H17 cells

The impact of T_H17 cells early in life, closely linked to Treg cells, and differences in maturation depending on maternal atopy are unknown. IL-17 secretion was significantly increased after all stimulations (besides phytohemagglutinin); however, at low levels ([Table III](#)), it was not significantly different depending on maternal atopy. Assessing correlations of IL-17 with other T-cell lineage cytokines, we detected significant increases of primarily T_H2 cytokines (IL-13, *r* > 0.6), and at lower levels also of T_H1 (IFN-γ) and proinflammatory cytokines (TNF-α) with IL-17 induction ([Table III](#); see this article's [Table E6](#) in the Online Repository at www.jacionline.org). Including maternal atopy, there were overall no significant changes in the correlations ([Table III](#)).

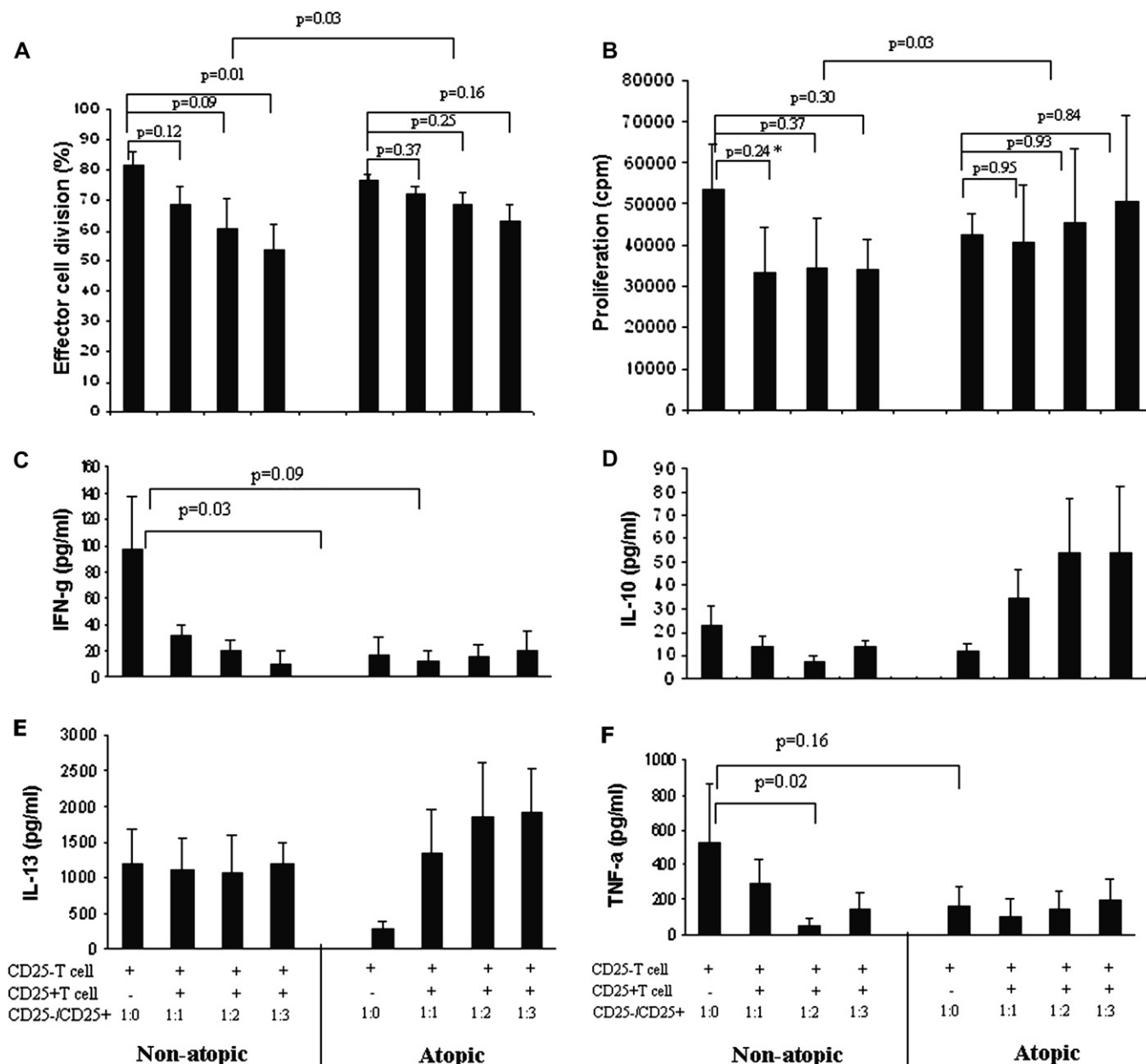


FIG 2. Division of effector T cells was shown in percentage, lymphocyte proliferation of effector T-cells in cpm, and cytokine secretion in pg/mL after stimulation and addition of CD4⁺CD25⁺ T cells in increasing doses (1:1 to 1:3). Division of effector T cells and lymphoproliferation were measured with CFSE staining and ³H-Thymidine incorporation, cytokine secretion by LUMINEX technology. Cord blood samples of N = 8 nonatopic and 6 atopic mothers were shown (A-F).

Correlations were not significantly influenced by outliers (see this article's Fig E4, A-F, in the Online Repository at www.jacionline.org). Including paternal atopy, similarly TNF-α and IL-17 correlations were significant, yet low without paternal atopy, but not with paternal atopy ($r = 0.34$; $P = .002$). Intracellular IL-17 protein and isolated T-cell IL-17 cytokine expression were low (~5%) and could consequently not be suppressed after Treg cell addition (not shown). There was no strong correlation between IL-17 and Treg cell-associated markers Foxp3, LAG3, or CTLA4. There were weak positive correlations between IL-17 and GITR for LpA-induced ($r = 0.44$; $P = .01$), peptidoglycan-

induced ($r = 0.39$; $P = .02$), and *D pteronyssinus* 1-induced ($r = 0.45$; $P = .02$) stimulations.

To assess interactions between Treg and T_H17 populations, different stimulation conditions were used (see the Results section of the Online Repository). After IL-6 stimulation, IL-17 cells increased at low percentages, whereas CD25⁺Foxp3⁺ cells were largely activated with TGF-β, but also with IL-6 and phytohemagglutinin stimulation alone (see this article's Fig E5 in the Online Repository at www.jacionline.org). Stimulation with IL-6 and TGF-β resulted in low IL-17. Proliferation was increased under all conditions. Thus, a balanced regulation of

TABLE III. IL-17 secretion in CBMCs after stimulation, correlation of IL-17 with T_H1/T_H2 cells in all CBMCs and CBMCs with (A) and without maternal atopy (NA)

Parameter	Stimulus	No maternal atopy (median ± IQR) N = 68	Maternal atopy (median ± IQR) N = 48	P value (comparison of NA-A)		
IL-17	PHA	0.6 (0.01/1.4)	1.6 (0.01/1.1)	.62		
	LpA	3.6 (0.9/6.4)*	2.0 (0.01/6.5)*	.44		
	Ppg	3.0 (0.8/10.8)*	3.6 (0.7/11.2)*	.93		
	D	2.8 (0.7/8.2)*	2.8 (1.0/8.5)*	.77		
	D+L	4.5 (2.5/10.8)*	5.5 (3.9/15.6)*	.43		
Correlation of cytokines after LpA stimulation		r	P	Correlation of cytokines after Ppg stimulation	r	P value
IL-17 and IL-13		0.70	<.0001	IL-17 and IL-13	0.64	<.0001
NA		0.68	<.0001	NA	0.61	<.0001
A		0.74	<.0001	A	0.68	<.0001
IL-17 and IL-5		0.32	.0006	IL-17 and IL-5	0.36	<.0001
NA		0.26	.03	NA	0.40	.0009
A		0.38	.009	A	0.31	.04
IL-17 and IFN-γ		0.34	<.0002	IL-17 and IFN-γ	0.35	<.0001
NA		0.30	.01	NA	0.31	.01
A		0.40	.005	A	0.43	.003

D, *D. pteronyssinus* 1; PHA, phytohemagglutinin; Ppg, peptidoglycan.Spearman's correlation coefficient *r*.**P* < .05, significant differences to unstimulated values per group.

CD4⁺CD25⁺Foxp3⁺ and T_H17 cells may take place already at an early stage.

In summary, T_H17 cells were not differently regulated depending on maternal atopy, but highly correlated with T_H2 cells. After distinct stimulation, CD25⁺IL-17⁺ cells were upregulated compared with CD25⁺Foxp3⁺ cells, potentially pointing to a balance between T_H17 cells and Treg cells at this early stage of maturation.

DISCUSSION

We have comprehensively analyzed the role of Treg cells in addition to T_H2/T_H1 cells in CBMCs of offspring from atopic/nonatopic mothers. We have identified 5 main important findings regarding early immune regulation after innate and adaptive/mitogen activation. First, the number of Treg cells, characterized as CD4⁺CD25⁺high T cells, was decreased in CBMCs with maternal atopy after innate stimulation. Second, CBMCs from atopic mothers had significantly lower expression of the Treg-associated markers GITR and LAG3, and trendwise lower expression for Foxp3 and CTLA4 after innate stimulation. Third, Treg cells were less efficient in mitogen-induced suppressive capacities of T-cell division, proliferation, and cytokine secretion in CBMCs of atopic mothers. This is supported by lower LpA-induced IL-10 and IFN-γ secretion in CBMCs from atopic mothers before suppression. Fourth, T_H17 cells as potential regulators beyond T_H1/T_H2/Treg lineages were present at this early stage of maturation, but remotely expressed in CBMCs, primarily after innate stimulation. IL-17 was highly correlated with IL-13 secretion, independent of maternal atopy. Fifth, paternal atopy and birth characteristics had no significant influence on the immunological outcomes. These data indicate a differential regulation of Treg cells primarily after innate stimulation depending on maternal atopic background and a potential role of T_H17 cells in early life, yet independently of maternal atopy.

Early life events seem to be particularly critical for the development of allergic diseases.¹³ Our data demonstrate lower

and less efficient Treg cells in cord blood of atopic mothers, indicating a potential mechanism by which early life immune modulation can occur and may influence further atopic disease development. Maternal atopy represents an intrauterine immune milieu for the unborn child, which—in combination with exposure to several immunological stimuli—may set the stage for later immune development. In addition, one may suppose a regulation through genetic differences from both mother and father. However, whereas maternal atopy revealed a strong influence on immune regulation in this study, paternal atopy did not. This is consistent with studies showing a more pronounced impact of maternal than paternal atopy on later development of the child's atopy.¹⁸ It also points to the intrauterine environment as a critical immune modulator.

To date, the intrauterine environment has been described to influence T_H1/T_H2 modulation in the context of allergic stimulation.¹ Our study adds another dimension by demonstrating Treg cells as 1 critical mediator in early immune maturation, and importantly after innate stimulation. After adaptive/mitogen stimulation, the effect was present but not as strong, which suggests the requirement of specific innate stimulation to shape a child's immune development. Interestingly, maternal atopy had an impact on Treg cell markers, IL-10 and T_H1, but not T_H2 cytokines after indirect innate activation. This may account for predominance of T_H1 lineages, but not of the potential default T_H2 pathway. Of note, subset analysis revealed that IFN-γ was expressed by CD4/CD8-T cells and to a low percentage by natural killer cells, IL-10 and IL-13 were expressed by CD4/CD8 T cells besides CD3⁺ cells (see the Results section of the Online Repository).

All Treg cell-associated markers, and most significantly innate-induced GITR and LAG3, were downregulated in CBMCs of atopic mothers. Although indirect innate stimulation of Treg cells will involve initial activation of dendritic cells, subsequent DC-Treg interactions will participate after innate stimulation. GITR, a member of the TNFR superfamily, is activated by glucocorticoid-induced-TNFR-related protein ligand (GITRL), involved in autoimmune/inflammatory processes and

overexpressed in Treg cells.¹⁹ Our data indicate that this regulation—present in early life—is modulated differently depending on maternal atopy. Evaluation of GITR, not entirely specific for Treg cells, is a measure of both effector and Treg cells, and GITR triggering can have several distinct effects either inhibiting or stimulating Treg cell activity. In the context of allergic diseases, GITR can modulate both T_H1/T_H2 -mediated inflammatory diseases and, for example, anti-GITR-mAb exacerbates murine ovalbumin-induced airway inflammation.²⁰ In this study in CBMCs, we did not specifically activate GITR but rather observed an increase of GITR along with Foxp3 and intriguingly LAG3. LAG3, an MHC class II-binding CD4 homolog, involved in expansion of activated T cells, was demonstrated to be required for maximal Treg cell activation in mice.²¹ LAG3 negatively regulates T-cell homeostasis by regulatory cell-dependent and independent mechanisms²¹ and modulates both *in vitro* and *in vivo* suppressive function of $CD4^+CD25^+$ T cells. Thus, one may speculate that LAG3 regulation may have an impact on regulatory capacities of T-cell differentiation during maturation.

Treg cells have constitutively high CTLA4 expression intracellular and low surface CTLA4 levels, which become upregulated on activation.²² In this study, CTLA4 was upregulated early in life and shows similar trends like the additional Treg cell marker with lower expression in CBMCs of atopic mothers. CTLA4 is an important negative regulator of T-cell activation to attenuate T-cell responses. Possibly, in early life, lower CTLA4 expression contributes to weaker attenuation of default immune responses, potentially contributing to development of allergies in childhood.

Although our data consistently point to less efficient Treg cell function with maternal atopy, the trend (and not significance) of lower Foxp3 expression was somewhat surprising. Although several Treg cell subsets with suppressor activity were described, Foxp3 is postulated as the most specific marker for $CD4^+CD25^+$ Treg cells. However, Gavin et al²³ detected that human Foxp3 expression could be induced transiently in non-Treg $CD4^+$ and $CD8^+$ T cells. This is in contrast to murine data, indicating differences between human and murine Foxp3 regulation. In our human study early in life, Foxp3 was also expressed in $CD4^+CD25^-$ cells, and Foxp3 expression in bulk culture was lower in cord blood with maternal atopy, but not significantly. Potentially, the number of samples may not be sufficient, or Treg cells characterized by Foxp3 mRNA are not truly differentially regulated depending on maternal atopy. However, this study examines a far larger cohort than most immunological studies, including our own previous study.^{5,6} Our findings most likely indicate that in maternal atopy offspring, Treg cells were differentially regulated, but assessment of Foxp3 mRNA alone and in correlation with IL-10 may not be the most specific and sufficient method for Treg cell evaluation. We could demonstrate Foxp3 expression on protein level, which is specific for Treg cells but also—in a small percentage—for $CD4^+CD25^-$ T cells. Thus, our present data support caution in using Foxp3 mRNA expression in bulk culture as a single parameter for Treg cell assessment, but rather support a selection of specific markers to profoundly analyze Treg cell function. One limitation of this study and 1 potential useful addition for future analysis may be to assess CD103/CD127 surface expression, recently described to differentiate Treg cell subsets independent of CD25 expression.^{24,25}

Correlations of T_H1/T_H2 lineages with IL-10/IL-17 may point to a regulatory pattern linked to additional T-cell populations.

Expanding the knowledge beyond T_H1/T_H2 lineages, we postulated that T_H17 cells may play a role in early life. In this study, low expression may either be a result of complex stimulation requirements or account for immature development of T_H17 cells, yet modest elevation after innate (and not adaptive/mitogen) immune stimulation may point to relevance in innate-induced subsequent maturation. No differences depending on maternal atopy may be attributable to immature regulation, rather than definitely indicating no difference in T_H17 cells. Correlations of IL-17 with primarily T_H2 and partly T_H1 cells but not IL-10 independent of atopy indicate a close regulation potentially not involving Treg cells. Low correlations of IL-17 with GITR/LAG3 but not Foxp3 or CTLA4 require more detailed assessment. However, already in CBMCs, differential regulation of Treg cells in balance with T_H17 cells could be crucial in modulating protective immune responses before disease development.

We are aware of some limitations of this study. First, we examined CBMCs and Treg cells of 2 groups of mothers with a heterogeneous phenotype. Maternal atopy includes 3 diseases that may be of distinct immunological origin. However, further sensible distinction was not feasible because of small numbers. Thus, we cannot draw conclusions of immune regulation regarding a specific maternal phenotype. Second, we examined some Treg cell-associated markers in bulk culture, and these may not always be entirely specific for Treg cells. However, the strength of this study is a detailed investigation of the early immune system at several levels, including functional analysis in a well documented population, all pointing to consistent findings. Also, this study includes a large group of 118 subjects selected by using strict inclusion and exclusion criteria.

In summary, neonates of atopic mothers are born with a lower amount and functional capacity of regulatory T cells in addition to lower T_H1 cells, potentially shifting early immune development toward an unfavorable proallergic immune pathway. Additional innate stimulation during maturation, likely involving T_H17 cells, may influence and balance healthy immune regulation. Longitudinal follow-up studies into childhood are underway for a better understanding of the balance and potentially protective capacity of Treg and T_H17 cells during maturation.

We thank the study participants and staff of the Department of Gynecology. We acknowledge Isolde Schleich and Gabriele Sulski for excellent technical assistance and Michaela Schedel for review and thoughtful critique of this manuscript.

Clinical implications: These data support a critical role for Treg cells in the modulation of early immune responses in relation to maternal atopy. T_H17 cells may be involved in immune regulation early in life, independently of maternal atopy.

REFERENCES

1. Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999; 353:196-200.
2. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;347:869-77.
3. Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998;160:4730-7.
4. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347:911-20.

5. Schaub B, Campo M, He H, Perkins D, Gillman MW, Gold DR, et al. Neonatal immune responses to TLR2 stimulation: influence of maternal atopy on Foxp3 and IL-10 expression. *Respir Res* 2006;7:40.
6. Amoudruz P, Holmlund U, Malmstrom V, Trollmo C, Bremme K, Scheynius A, et al. Neonatal immune responses to microbial stimuli: is there an influence of maternal allergy? *J Allergy Clin Immunol* 2005;115:1304-10.
7. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 2006;24:677-88.
8. Ivanov S, Bozinovski S, Bossios A, Valadi H, Vlahos R, Malmhall C, et al. Functional relevance of the IL-23-IL-17 axis in lungs in vivo. *Am J Respir Cell Mol Biol* 2007;36:442-51.
9. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;358:1129-33.
10. Velasco G, Campo M, Manrique OJ, Bellou A, He H, Arestides RS, et al. Toll-like receptor 4 or 2 agonists decrease allergic inflammation. *Am J Respir Cell Mol Biol* 2005;32:218-24.
11. Schaub B, Bellou A, Gibbons FK, Velasco G, Campo M, He H, et al. TLR2 and TLR4 stimulation differentially induce cytokine secretion in human neonatal, adult, and murine mononuclear cells. *J Interferon Cytokine Res* 2004;24:543-52.
12. Lauener RP, Birchler T, Adamski J, Braun-Fahrlander C, Bufe A, Herz U, et al. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. *Lancet* 2002;360:465-6.
13. Ege MJ, Bieli C, Frei R, van Strien RT, Riedler J, Ublagger E, et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol* 2006;117:817-23.
14. Gold DR, Willwerth BM, Tantisira KG, Finn PW, Schaub B, Perkins DL, et al. Associations of cord blood fatty acids with lymphocyte proliferation, IL-13, and IFN-gamma. *J Allergy Clin Immunol* 2006;117:931-8.
15. Schaub B, Tantisira KG, Gibbons FK, He H, Litonjua AA, Gillman MW, et al. Fetal cord blood: aspects of heightened immune responses. *J Clin Immunol* 2005;25:329-37.
16. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986-94.
17. Heinrich J, Richter K, Frye C, Meyer I, Wölke G, Wjst M, et al. ECRHS, results and the contribution of the two German study centers. *Pneumologie* 2002;56:297-303.
18. Wright AL. The epidemiology of the atopic child: who is at risk for what? *J Allergy Clin Immunol* 2004;113:S2-7.
19. Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* 2002;3:135-42.
20. Patel M, Xu D, Kewin P, Choo-Kang B, McSharry C, Thomson NC, et al. Glucocorticoid-induced TNFR family-related protein (GITR) activation exacerbates murine asthma and collagen-induced arthritis. *Eur J Immunol* 2005;35:3581-90.
21. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. *Immunity* 2004;21:503-13.
22. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 2000;192:295-302.
23. Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, et al. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 2007;445:771-5.
24. Allakhverdi Z, Fitzpatrick D, Boisvert A, Baba N, Bouguermouh S, Sarfati M, et al. Expression of CD103 identifies human regulatory T-cell subsets. *J Allergy Clin Immunol* 2006;118:1342-9.
25. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006;203:1701-11.

METHODS

Study population

Umbilical cord blood was obtained at the time of delivery from healthy neonates born at term after uncomplicated pregnancies. We determined maternal and paternal atopy as presence or absence of an allergic illness as assessed by a doctor's diagnosis of asthma and/or eczema and/or hay fever. In addition, we measured total and specific IgE (RAST). A positive specific IgE was defined as 1 or more positive reactions ≥ 0.7 IU/mL to a panel of 20 common allergens, which included the most common aeroallergens (mites, *D pteronyssinus* and *Dermatophagoides farinae*; birch pollen; hazelnut pollen; timothy grass; mugwort; plantain), animals (cat, horse, dog), *Alternaria*, and food including egg white, milk protein, peanut, hazelnut, carrot, wheat flour, and soy bean.

Maternal smoking status was determined at delivery: the mother was asked if she smoked at all, until she was aware of the pregnancy, or during pregnancy, and how many cigarettes a day. The 5-minute Apgar score (assigning a score of 0-2 to heart rate, respiratory effort, muscle tone, reflex irritability, and color) was abstracted from birth records and noted as normal above 8. Neonatal race was determined on the basis of parental report of race in the questionnaire.

Exclusion criteria ($n = 43$ excluded) were maternal or neonatal problems such as multiple gestation, chronic diseases of the mother, or inability to answer questions in German. Specifically, mothers with autoimmune or rheumatoid diseases ($n = 3$), infection in the last trimester, and fever around birth or use of chronic medications during pregnancy were excluded. In addition, neonates with pathological cardiocardiography ($n = 4$), asphyxia ($n = 2$), infected amniotic fluid ($n = 6$), and signs of inflammation ($n = 22$) assessed clinically and by objective measurements such as IL-6 and C reactive protein were excluded. For analysis, data were excluded ($n = 6$) if differences from laboratory standards were detected.

Three neonates with minor growth retardation below 3000 g, otherwise healthy, were included in the study. Otherwise, subjects were included when data about proliferation, cytokine secretion, and gene expression were complete.

Lymphocyte proliferation, cytokine secretion of CBMCs

Cord blood was collected from the umbilical vein after delivery and processed within 24 hours. CBMCs were isolated by density-gradient centrifugation with Ficoll-Hypaque Plus (Amersham Bioscience, Uppsala, Sweden) after dilution in PBS (Gibco, Karlsruhe, Germany; Invitrogen, Karlsruhe, Germany). Cells, washed in RPMI 1640, were diluted in 10% human serum (Sigma Aldrich, Taufkirchen, 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 LpA (*Salmonella* Minnesota Re-595, Re mutant; $0.1 \mu\text{g/mL}$), peptidoglycan (*Staphylococcus aureus*; $10 \mu\text{g/mL}$), phytohemagglutinin ($5 \mu\text{g/mL}$; all Sigma Aldrich) and *D pteronyssinus* 1 ($30 \mu\text{g/mL}$; Indoor Biotechnologies, Charlottesville, Va) or a combination of *D pteronyssinus* 1 and LpA (D+L) for 3 days and compared with unstimulated cells. Endotoxin concentrations in peptidoglycan, *D pteronyssinus* 1, and phytohemagglutinin, measured by Limulus assay, were low (<0.01 EU/m = 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 LpA (0.01 – 100 ng/mL), we detected increased lymphocyte proliferation with doses $>1 \text{ ng/mL}$; therefore, levels of $<0.01 \text{ ng/mL}$ had no influence on the analysis. After incubation at 37°C in a humidified 5% CO_2 chamber, samples were pulsed with $1 \mu\text{Ci } ^3\text{H}$ -Thymidine for additional 8 hours. Cells, harvested with a ComBI cell harvester (Skatron Instrument, Lier, Norway) onto filter plates, were read using a β -Counter. Proliferation was assessed by cpm and quantified by SI, which was calculated as the ratio of mean cpm of stimulated over unstimulated replicates.

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

Real-time quantitative RT-PCR

Total RNA ($1 \mu\text{g}$), isolated with TRI Reagent, was processed with reverse transcriptase (Invitrogen). For RT-PCR, we used $0.34 \mu\text{L}$ cDNA, $7.5 \mu\text{L}$ primer, $9.66 \mu\text{L}$ diethyl pyrocarbonate water, and $12.5 \mu\text{L}$ SYBR Green Master-Mix/well (total $30 \mu\text{L}$; Applied Biosystems, Darmstadt, Germany). Gene-specific PCR products were measured continuously by means of iCycler iQ-multicolor Real-Time PCR-Detection-System (Bio-Rad, Munich, Germany) 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 expected size of the PCR fragment. mRNA-specific oligonucleotide primers (forward/reverse) were designed with Vector NTI-Advance10 (Invitrogen): 18S: 5'-AGTCCCTGCCCTTTGTACACA-3', 5'-GATCCGAGGGCCTCACTAAAC-3'; Foxp3: 5'-ACCTTCCCAAATCCAGTGC-3', 5'-GAA GATGGTCCGCCTGGC-3'; LAG3: 5'-CAATGGCGACTTTACCTTC-3', 5'-CCTCTGGGATGGGGTGTC-3'; GITR: 5'-GCACCACCCTTGTCCTCC-3', 5'-CAGCGTTGTGGGTCTTGTTC-3'; CTLA4: 5'-TGGCC CTGCACTCTCTGT-3', 5'-GGACCTCAGTGGCTTTGCCT-3'; TGF- β : 5'-ACAAGTTCAAGCAGAGTACACACAGC-3', 5'-CACTGCCGCACAA CTCCG-3'.

Flow cytometry

For surface staining, CBMCs were incubated in aliquots of 5×10^5 cells ($100 \mu\text{L}$ $1 \times \text{PBS}$, 5% FCS, 0.5% Tween), with saturating concentrations of fluorochrome-labeled antibodies for 30 minutes at 4°C . The samples were washed twice with 1 mL buffer and resuspended in $400 \mu\text{L}$ PBS-FCS. For intracellular Foxp3 staining, cells were stained with $8 \mu\text{L}$ antihuman CD4-FITC, and $4 \mu\text{L}$ antihuman CD25-RPE-Cy5 antibodies per tube ($1 \times 10^6/100 \text{ mL}$). After resuspension, 1 mL 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 1X Permeabilization Buffer. After blockade with 2% normal rat serum ($2 \mu\text{L}$) in 1X Permeabilization Buffer at 4°C for 15 minutes, $10 \mu\text{L}$ antihuman Foxp3-phycoerythrin antibody or $5 \mu\text{L}$ mouse IgG $_{2a}$ -phycoerythrin isotype control in 1X 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.

For intracellular IL-17 staining, cells were cultured with $25\text{-}\mu\text{L}$ beads for $1 \times 10^6/1 \text{ mL}$ cells, antihuman CD3/CD28 T-cell expander beads (DynaL Biotech GmbH, Hamburg, Germany), recombinant human IL-1 β (20 ng/mL), and recombinant human IL-6 (100 ng/mL ; R&D Systems, Wiesbaden, Germany). In optimization experiments, TGF- β was added in increasing doses (100 ng/mL) for comparison. After culture for 72 hours, cells were washed and beads removed with the magnet. Cells were restimulated for 5 hours with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma Aldrich), $1 \mu\text{L/mL}$ Ionomycin (Sigma Aldrich), and $2 \mu\text{L/mL}$ monensin (eBioscience NatuTec). Then intracellular antihuman IL-17 ALEXA antibody ($10 \mu\text{L}$ in 1×10^6 cells/ 100 mL) or corresponding IgG $_{1\kappa}$ isotype control (eBioscience NatuTec) was added and processed as described for Foxp3 staining. The isotype control was always negative.

Functional analysis of Treg cells

After isolation of CD3 $^+$ cells with the CD3 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), CD3 $^+$ cells were irradiated with 30 Gy (3000 rad , 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 above 95%. CD4 $^+$ CD25 $^-$ T cells were labeled with $5 \mu\text{mol/L}$ 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 /well) were incubated with irradiated CD3 $^+$ cells (4×10^4 /well) in coculture with or without CD4 $^+$ CD25 $^+$ T cells (2×10^4 /well). Results were compared before and after stimulation with $0.8 \mu\text{g/mL}$ phytohemagglutinin. After 3 days

of culture, division of CD4⁺CD25⁻ T cells was analyzed by using CellQuest software (Becton Dickinson, Heidelberg, Germany). The proliferation of CD4⁺CD25⁻ T cells was assessed by ³H-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).

RESULTS

Population characteristics

From the 48 mothers with atopy, 9 mothers (18.8%) had asthma and were currently not on systemic corticosteroids. Thirty-six mothers (75%) had hay fever, and 11 (22.9%) had eczema. Three mothers had asthma and hay fever, 1 mother had asthma and atopic eczema, another mother had hay fever and atopic eczema, and 1 woman had all 3 conditions. There were 7 mothers smoking a mean of 6.5 (nonatopic) or 5.7 (atopic) cigarettes per day during pregnancy, not significantly different between groups.

Higher GTR and LAG3 but not Foxp3 expression in CBMCs without maternal atopy.

LAG3 was the only examined gene that was mildly influenced in part by paternal atopy. When analyzing data with the 2 most distinct groups, either no paternal atopy or both maternal and paternal atopy, the following change in gene expression data was observed in comparison to analysis with no atopy compared with maternal atopy only: phytohemagglutinin, no atopy versus both atopy, median, 280.6 versus 63.6, $P = .16$ (no atopy/maternal atopy, 216.7 vs 150.0, $P = .31$); LpA, 3.4 versus 2.0, $P = .35$ (no atopy/maternal atopy 3.5 vs 1.1, $P = .06$), and D+L, no atopy versus atopy, median, 7.0 versus 3.0, $P = .003$ (no atopy/maternal atopy, 6.6 vs 1.4, $P = .07$).

Correlations of cytokine secretions and Treg cell-associated genes

When assessing correlations of cytokine data with maternal or paternal atopy separately, correlations did not change significantly (not shown). When assessing correlations with either both

atopic or no atopic parents, significant correlations were observed; for instance, the correlation between LpA-induced IFN- γ and IL-5 was significant only when both or 1 of the parents were atopic, but not if both were nonatopic (Table E2). For peptidoglycan-induced IFN- γ /IL-13, IFN- γ /IL-5, IL-13/TNF- α , and IL-5/TNF- α , correlations were significant only in CBMCs of both non-atopic parents.

The strength of the correlation was above 0.6 for LpA-induced IL-5/IL-13 and IL-5/TNF- α and IL-13/TNF- α and IL-13/IFN- γ . Atopy had no significant influence on the correlation. For peptidoglycan-induced cytokines, correlations were increased ($r > 0.6$) for IL-10/IL-5 and IL-13/IL-5 with both atopic parents. Thus, parental atopy seems to change correlations of T_H1/T_H2 cytokines after different Toll-like receptor stimulating conditions.

In a subset of 4 samples, a selection of cytokine-secreting cells were analyzed by using intracellular staining by flow cytometry. A differentiation of data in CBMCs of atopic and nonatopic mothers was not feasible because of the number of samples. IFN- γ was produced by T cells (CD4 and CD8) and natural killer cells, partly by CD3⁻ cells. IL-10 was produced by CD4 cells, including the subgroup of CD4CD25 high cells and CD8 cells, whereas IL-13 was produced by CD4 and CD8 and partly by CD3⁻ cells.

In detail, IFN- γ -positive cells in the CBMC population were 5.36% (mean) after LpA stimulation (7.62% in unstimulated conditions); IFN- γ ⁺CD4⁺ in CBMCs accounted for 0.4%, IFN- γ ⁺CD8⁺ cells were 0.26%, and IFN- γ ⁺ natural killer cells (CD3⁻CD56⁺) were 0.25%. The other IFN- γ ⁺ cells were CD3⁻.

IL-10-positive cells in the CBMC population were 4.86% after LpA stimulation (5.31 in unstimulated cells). IL-10 CD4⁺ cells accounted for 1.02%, IL-10 CD8⁺ cells for 0.54%. CD4⁺CD25⁺IL-10⁺ cells were 0.47%, and the CD4CD5⁺high IL-10 cells were 0.34%.

IL-13-positive cells in the CBMC population were 7.07% after LpA stimulation (4.23% in unstimulated cells); IL-13 CD4⁺ cells accounted for 3.02%, IL-10 CD8⁺ cells for 1.34%. Results indicated 1.91% were CD3⁻ cells.

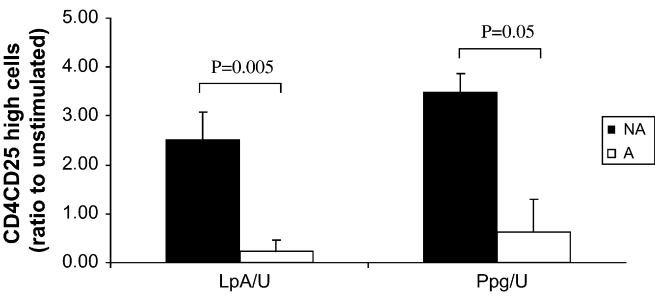


FIG E1. CD4⁺CD25 high T cells (ratio of stimulated vs unstimulated cells). CD4⁺CD25 high T cells were significantly higher in LpA/U and peptidoglycan (*Ppg*)/U samples in CBMCs of nonatopic compared with atopic mothers. CD4⁺CD25 high T cells were assessed by flow cytometry. Data are shown as means $\pm$ SEMs. A, Atopic; NA, nonatopic; U, unstimulated.

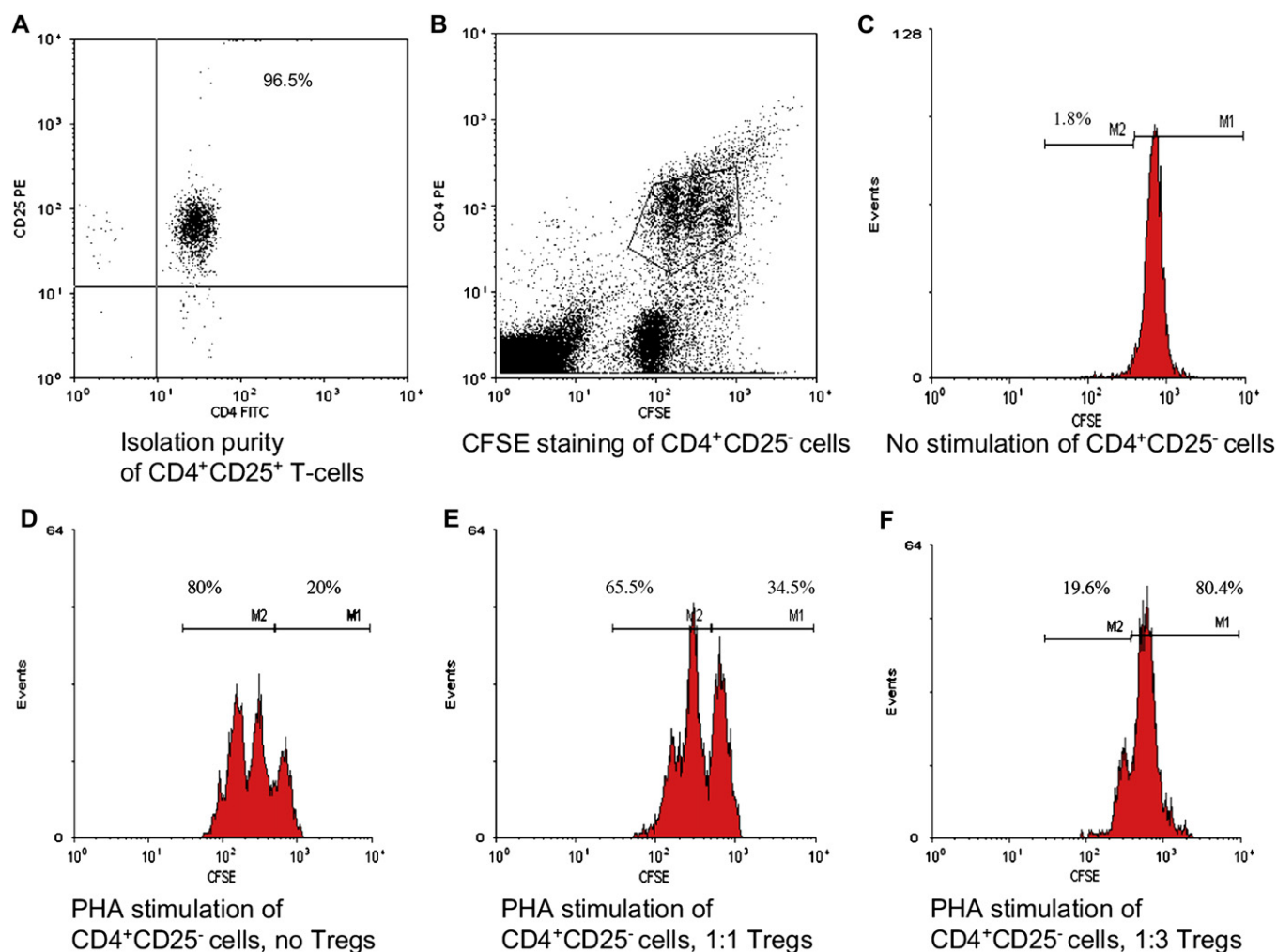


FIG E2. Suppressive capacity of CD4⁺CD25⁺ T cells. CD4⁺CD25⁺ T cells significantly suppressed effector T cells after mitogen stimulation in a dose-dependent manner. **A**, Purity of isolated CD4⁺CD25⁺ T cells. **B**, CFSE staining of effector T cells. **C**, Unstimulated effector T cells showed no significant T-cell division, 1.8% (CFSE staining). **D**, Significant T-cell division after phytohemagglutinin (PHA) stimulation (80%). **E** and **F**, Dose-dependent suppression of effector T cells through CD4⁺CD25⁺ T cells (65.5% and 19.6% T-cell division). **A-F**, CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were isolated as described in Methods. CD4⁺CD25⁻ cells were labeled with CFSE and incubated with irradiated CD3⁺ T cells in coculture without or with increasing ratios of CD4⁺CD25⁺ T cells (1:1 to 1:3). The division of CD4⁺CD25⁻ T cells was measured before and after 3 days of stimulation with 0.8 μ g/mL PHA with flow cytometry. Measurement and analysis were performed by using FACSscan and CellQuest software. One representative experiment of 14 is shown.

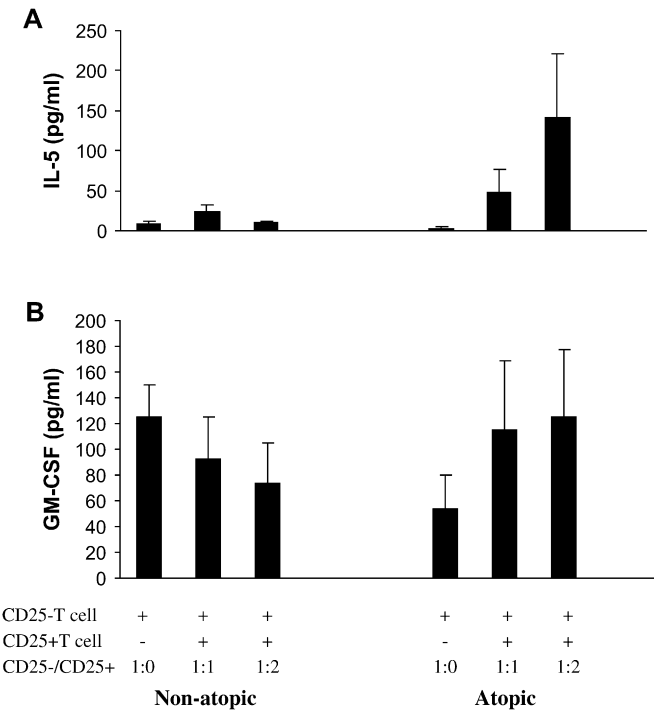


FIG E3. Suppression of T-cell cytokine secretion of IL-5 and GM-CSF. **A** and **B**, Cytokine secretion of effector T cells of GM-CSF but not IL-5 was decreased after suppression through CD4⁺CD25⁺ T cells in a dose-dependent manner in cord blood from nonatopic mothers. Isolation and suppression assays were described in Methods; cytokine secretion was assessed by LUMINEX technology. Mean or median ($\pm$ SEM) values of cord samples of N = 8 nonatopic and 6 atopic mothers are shown.

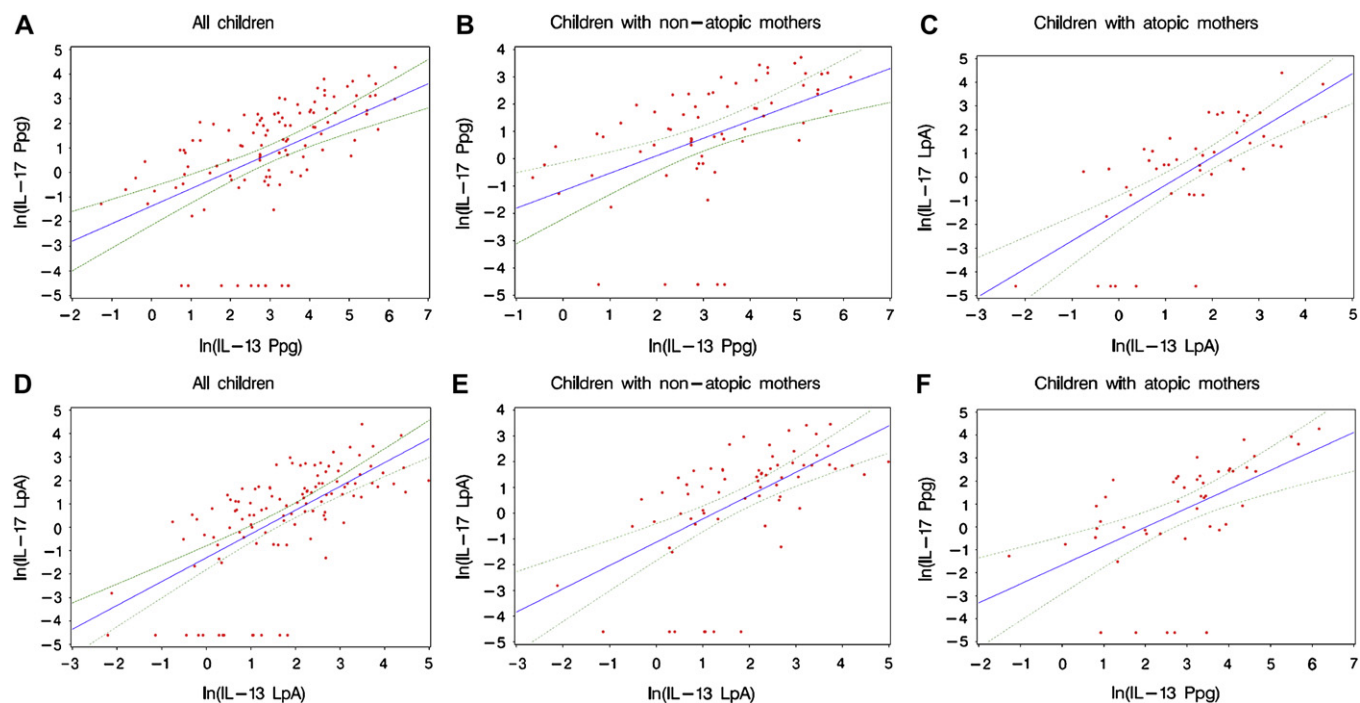


FIG E4. A-F, Correlation of LpA and peptidoglycan (Ppg)-induced secretion of IL-17 and IL-13 in CBMCs of all children and of children without and with atopic mothers. Cytokines (pg/mL) were measured by LUMINEX. Data are shown after log-transformation with regression line and CI for mean predicted values. *ln*, Log transformation.

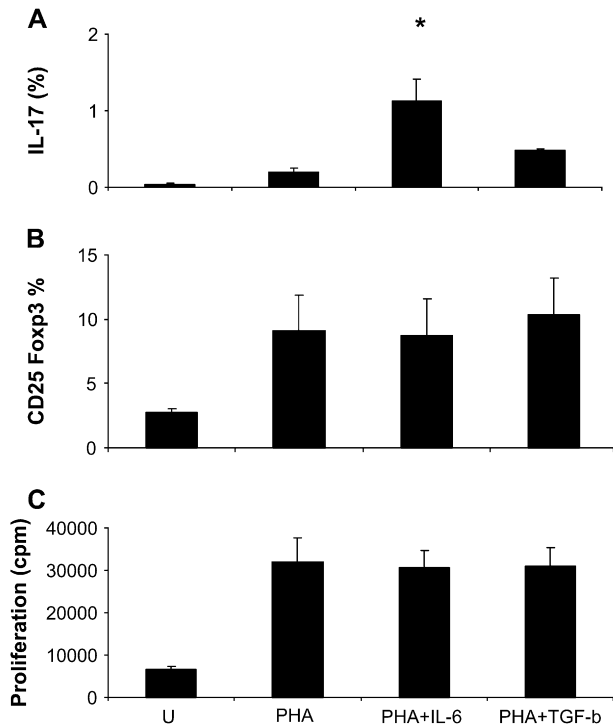


FIG E5. A-C, CD25⁺ IL-17 positive cells and CD25Foxp3 cells. Intracellular IL-17, Foxp3⁺ expression, and proliferation after stimulation with phytohemagglutinin (PHA) with and without IL-6 and TGF- β . CBMCs were stimulated, and intracellular IL-17 or Foxp3 was assessed as described in Methods. Proliferation in cpm was measured by ³H-Thymidine incorporation. Data are shown as mean ($\pm$ SEM) of $n = 10$ experiments. * $P > .05$. U, Unstimulated.

TABLE E1. Correlation coefficients of Treg cell-associated genes

Correlation of stimulated GITR with LAG3			Correlation of stimulated GITR with Foxp3			Correlation of stimulated LAG3 with Foxp3		
	<i>r</i> *	<i>P</i> value		<i>r</i> *	<i>P</i> value		<i>r</i> *	<i>P</i> value
PHA all	0.61	<.0001	PHA all	0.49	.002	PHA all	0.53	<.0001
NA	0.66	<.0001	NA	0.74	<.0001	NA	0.75	<.0001
A	0.54	.02	A	0.22	.41	A	0.29	.25
LpA all	0.40	.04	LpA all	0.33	.08	LpA all	0.02	.88
NA	0.09	.73	NA	0.36	.18	NA	−0.10	.70
A	0.53	.08	A	−0.24	.44	A	−0.11	.71
Ppg all	0.27	.15	Ppg all	0.28	.16	Ppg all	0.16	.44
NA	0.14	.63	NA	0.03	.90	NA	0.54	.08
A	−0.12	.65	A	0.28	.33	A	0.04	.88
D all	0.55	.004	D all	0.59	<.0001	D all	0.26	.17
NA	0.80	<.0001	NA	0.58	.01	NA	0.46	.09
A	0.31	.32	A	0.53	.11	A	0.04	.88
D+L all	0.72	.0001	D+L all	0.69	<.0001	D+L all	0.58	.001
NA	0.49	.21	NA	0.68	.02	NA	0.83	.005
A	0.79	.01	A	0.69	.04	A	0.42	.21

A, CBMCs from offspring of atopic mothers; D, *D pteronyssinus* 1; NA, CBMCs from offspring of nonatopic mothers; PHA, phytohemagglutinin; Ppg, peptidoglycan.

Data in **boldface** are significant.

*Spearman rank test.

TABLE E2. Lymphocyte proliferation and cytokine secretion (pg/mL) after stimulation with mitogen phytohemagglutinin (PHA), the innate stimuli LpA and peptidoglycan (Ppg), or allergen Der p 1, respectively, compared with unstimulated CBMCs (U)

Parameter	Stimulus	No maternal atopy (median ± IQR)	Maternal atopy (median ± IQR)	P value† (comparison of NA-A)
IL-2	PHA	0.01 (0.01/1.39)**	0.01 (0.01/0.94)**	.90
	LpA	0.01 (0.01/3.88)**	0.01 (0.01/3.78)**	.63
	Ppg	0.01 (0.01/3.88)**	0.01 (0.01/3.99)**	1.00
	D	0.01 (0.01/4.09)**	0.01 (0.01/5.60)**	.26
	D+L	0.01 (0.01/0.01)	0.01 (0.01/0.01)	.72
IL-12	PHA	1.63 (1.04/2.76)**	1.73 (1.05/2.61)*	.86
	LpA	1.07 (0.52/1.62)*	1.10 (0.67/1.83)	.70
	Ppg	0.75 (0.25/2.04)	0.96 (0.38/1.38)	.69
	D	1.44 (0.89/2.21)**	1.52 (0.82/2.01)	.64
	D+L	1.53 (1.06/2.06)**	1.67 (1.01/2.12)*	.61
TNF-α	PHA	704.9 (214.0/1805.7)**	761.4 (300.3/1767.7)**	.82
	LpA	1066.4 (542.1/2234.7)**	1294.5 (433.9/1970.0)**	.90
	Ppg	2549.9 (1355.7/3635.8)**	2435.9 (1364.1/3479.1)**	.88
	D	1095.2 (631.2/2438.4)**	1319.3 (798.6/3060.3)**	.51
	D+L	913.9 (445.7/1655.1)**	1323.4 (505.4/3008.3)**	.25
IL-6	PHA	30,454 (21,227/180,000)**	27,267 (17,526/180,000)**	.39
	LpA	30,000 (18,053/51,748)**	30,000 (13,700/108,110)**	.96
	Ppg	25,568 (13,797/47,337)**	31,887 (12,564/180,000)**	.39
	D	30,000 (17,859/158,325)**	37,651 (27,445/180,000)**	.36
	D+L	30,000 (17,859/158,325)**	38,104 (15,277/302,539)**	.71
IL-13	PHA	559.93 (68.02/2553.73)**	911.57 (126.97/2699.96)**	.44
	LpA	8.78 (2.51/16.04)**	5.65 (1.91/14.42)**	.34
	Ppg	22.07 (9.51/66.79)**	25.40 (5.87/51.017)**	.56
	D	10.46 (4.30/35.50)**	11.13 (3.40/24.84)**	.82
	D+L	7.03 (2.99/17.90)**	12.12 (5.38/21.60)**	.23
IL-5	PHA	54.2 (22.9/92.1)**	29.2 (16.8/64.7)**	.25
	LpA	8.7 (3.4/26.2)**	6.5 (3.4/15.3)**	.34
	Ppg	34.3 (8.5/81.7)**	23.5 (6.1/46.7)**	.25
	D	11.9 (6.6/32.5)**	11.5 (6.2/16.7)**	.43
	D+L	10.1 (5.6/23.2)**	9.6 (7.7/19.5)**	.75
IL-4 (n = 10/5)	PHA	1.6 (0.6/3.4)*	1.0 (0.8/2.1)	.89
	LpA	0.2 (0.01/0.5)	0.4 (0.1/0.5)	.42
	Ppg	0.96 (0.3/1.9)*	3.6 (0.4/8.4)	.38
	D	0.2 (0.03/1.4)	1.9 (0.8/2.8)	.07
	D+L	0.3 (0.01/0.5)	9.1 (9.1/9.1)	.54
GM-CSF	PHA	6.9 (0.01/103.3)**	17.6 (0.7/83.5)**	.31
	LpA	1.6 (0.01/23.3)**	6.34 (0.01/27.1)**	.83
	Ppg	21.0 (0.01/338.5)**	77.8 (2.0/247.2)**	.84
	D	15.7 (0.01/10.2)**	44.3 (1.4/139.5)**	.15
	D+L	0.01 (0.01/20.36)**	3.236 (0.01/104.6)**	.22

D, *D pteronyssinus* 1; IQR, interquartile range.* $P \leq .05$; ** $P \leq .001$, Mann-Whitney rank-sum test. Comparison between stimulated and unstimulated data.

†Comparison between CBMCs of nonatopic (NA) and atopic (A) mothers with Mann-Whitney rank-test.

TABLE E3. Two groups of CBMCs with and without maternal atopy*: cytokine secretion (pg/mL) after stimulation with mitogen phytohemagglutinin (*PHA*), the innate stimuli LpA and peptidoglycan (*Ppg*), or allergen *D pteronyssinus* 1 (*D*) after subtraction of unstimulated values (*U*)

Parameter	Stimulus	No maternal atopy (NA) (mean ± SEM or median ± IQR)	Maternal atopy (A) (mean ± SEM or median ± IQR)	P value (comparison of NA-A)
IFN-γ – U	PHA	130.14 (18.38/264.58)**	95.71 (12.45/160.33)**	.33
	LpA	23.67 (3.75/127.58)**	12.37 (1.56/66.83)**	.04
	Ppg	27.63 (9.99/137.74)**	22.15 (5.00/80.67)**	.14
	D	42.14 (5.62/160)**	18.40 (4.53/118.61)**	.12
	D+L	130.13 (16.27/164.39)**	63.41 (9.05/151.31)**	.27
IL-10 – U	PHA	141.30 (62.74/234.77)**	102.95 (55.14/215.75)**	.32
	LpA	197.25 (128.20/350.32)**	167.00 (101.76/236.22)**	.057
	Ppg	785.79 (171.60/1445.74)**	549.63 (49.83/1221.83)**	.37
	D	199.37 (109.23/387.43)**	178.81 (90.47/263.04)**	.13
	D+L	290.23 (204.51/471.27)**	227.98 (153.31/352.87)**	.09

Data in *boldface* are significant.

*Maternal history of atopy was defined as doctor's diagnosis of asthma, hay fever, or eczema.

***P* ≤ .001.

TABLE E4. Correlations of T-cell cytokines in all children, stratified for double parental atopy (both atopic [A] or both nonatopic [NA])

Correlation of cytokines after LpA stimulation			Correlation of cytokines after peptidoglycan stimulation		
	<i>r</i>	<i>P</i> value		<i>r</i>	<i>P</i> value
IL-10 and IL-13	0.34	.0002	IL-10 and IL-13	0.48	<.0001
Both A	0.52	.02	Both A	0.60	.004
Both NA	0.21	.12	Both NA	0.47	.0005
IL-10 and IL-5	0.45	.0001	IL-10 and IL-5	0.64	<.0001
Both A	0.55	.01	Both A	0.72	.0002
Both NA	0.30	.03	Both NA	0.72	<.0001
IL-10 and IFN- γ	0.34	.0002	IL-10 and IFN- γ	0.09	.34
Both A	0.48	.03	Both A	-0.001	.99
Both NA	0.29	.04	Both NA	0.09	.49
IL-10 and TNF- α	0.34	.0002	IL-10 and TNF- α	0.18	.05
Both A	0.22	.34	Both A	0.07	.78
Both NA	0.18	.21	Both NA	0.08	.60
IFN- γ and TNF- α	0.28	.002	IFN- γ and TNF- α	0.29	.002
Both A	0.37	.09	Both A	0.11	.64
Both NA	0.19	.18	Both NA	0.24	.09
IFN- γ and IL-13	0.51	<.0001	IFN- γ and IL-13	0.44	<.0001
Both A	0.63	.002	Both A	0.27	.24
Both NA	0.39	.004	Both NA	0.33	.02
IFN- γ and IL-5	0.35	.0001	IFN- γ and IL-5	0.39	.007
Both A	0.57	.007	Both A	0.10	.64
Both NA	0.25	.07	Both NA	0.37	.008
IL-13 and TNF- α	0.61	<.0001	IL-13 and TNF- α	0.50	<.0001
Both A	0.53	.01	Both A	0.42	.06
Both NA	0.70	<.0001	Both NA	0.60	<.0001
IL-13 and IL-5	0.76	<.0001	IL-13 and IL-5	0.80	<.0001
Both A	0.75	<.0001	Both A	0.84	<.0001
Both NA	0.68	<.0001	Both NA	0.83	<.0001
IL-5 and TNF- α	0.60	<.0001	IL-5 and TNF- α	0.43	<.0001
Both A	0.58	.005	Both A	0.10	.65
Both NA	0.60	<.0001	Both NA	0.37	.008

r, Spearman correlation coefficient.

Data in *boldface* are significant.

N = 115 children; first line of each square always all children.

TABLE E5. Correlation of cytokines with Treg cell-associated genes

Correlations of cytokines with Treg cell-associated genes	Stimulus	<i>r</i>	<i>P</i> value
IL-10 with Foxp3	PHA	0.08	.59
	LpA	0.04	.82
	Ppg	−0.07	.68
	D	0.09	.59
	D+L	−0.01	.94
IL-10 with GITR	PHA	0.34	.03
	LpA	0.27	.14
	Ppg	0.05	.78
	D	0.12	.53
	D+L	0.07	.77
IL-10 with LAG3	PHA	0.38	.02
	LpA	−0.09	.63
	Ppg	−0.02	.91
	D	0.17	.33
	D+L	0.33	.17

D, *D pteronyssinus* 1; *PHA*, phytohemagglutinin; *Ppg*, peptidoglycan.

Data in *boldface* are significant.

r, Spearman correlation coefficient.

TABLE E6. Correlation of IL-17 cells with T_H1/T_H2 cells in all CBMCs and CBMCs with (A) and without maternal atopy (NA)

Correlation of cytokines after LpA stimulation	<i>r</i>	<i>P</i> value	Correlation of cytokines after peptidoglycan stimulation	<i>r</i>	<i>P</i> value
IL-17 and TNF-α	0.28	.002	IL-17 and TNF-α	0.41	<.0001
NA	0.33	.006	NA	0.48	<.0001
A	0.20	.17	A	0.32	.03
IL-17 and IL-10	-0.06	.50	IL-10 and IL-17	0.01	.87
NA	-0.06	.59	NA	-0.02	.84
A	-0.07	.66	A	0.09	.55

r, Spearman correlation coefficient.

Data in *boldface* are significant.