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EPIDEMIOLOGY AND GENETICS

Cord blood Tregs with stable *FOXP3* expression are influenced by prenatal environment and associated with atopic dermatitis at the age of one year

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Abstract

Background: Regulatory T cells (Tregs) with stable *FOXP3* expression are characterized by a specific demethylated region in the *FOXP3* gene (Treg-specific demethylated region, TSDR). The aim of this study was to analyse the influence of prenatal factors on cord blood Treg numbers, as detected by changes in the TSDR demethylation, and the subsequent risk for allergic diseases.

Methods: Analyses were performed within the LINA study in blood samples from pregnant women (34th gestational week) and in cord blood ($n = 346$ mother–child pairs). Treg numbers were detected via DNA demethylation in the *FOXP3* TSDR. At age 1, total and specific IgE was measured in children's blood. In addition, maternal cytokine production (Th1/Th2/Th17) was analysed. Exposure and disease outcomes were assessed by questionnaires.

Results: Boys had lower Treg numbers compared with girls ($P < 0.001$). Parental atopy history, particularly maternal hay fever and paternal asthma were related to lower Treg numbers in cord blood (adj. MR = 0.81, 95% CI = 0.68–0.97; adj. MR = 0.60, 95% CI = 0.45–0.81). Maternal cytokines (IL-13, IL-17E and IFN- γ) and maternal smoking/exposure to tobacco smoke during pregnancy were also associated with decreased cord blood Treg numbers (adj. MR = 0.89, 95% CI = 0.97–1.00). Children with lower Treg numbers at birth had a higher risk to develop atopic dermatitis (adj. OR = 1.55, 95% CI = 1.00–2.41) and sensitization to food allergens (adj. OR = 1.55, 95% CI = 1.06–2.25) during the first year of life.

Conclusions: These results indicate that both genetic and environmental factors presumably influence the development of foetal Tregs. Low cord blood Treg numbers may predict early atopic dermatitis.

As allergic diseases start to develop very early in life, it has been proposed that genetic predisposition as well as multiple prenatal factors [e.g. *in utero* microenvironment, maternal smoking and exposure to environmental tobacco smoke (ETS), diet] influence the *in utero* programming of the foetal immune system and therefore the susceptibility to develop allergic diseases (1–3). Regulatory T cells (Tregs) play an essential role in immune regulation of atopic diseases (4).

It has been hypothesized that *in utero* induced impairment in these regulatory pathways may play a key role in providing the basis for an allergy skewed phenotype. However, data concerning the relevance of *in utero* events for the imprinting of Tregs and children's atopic predisposition is still limited and contradictory. It has been suggested that Tregs are impaired in neonates with hereditary allergy risk (5, 6). In contrast, Rindsjö et al. (7) did not detect an effect of maternal

allergic disease on Treg subsets in cord blood. These inconsistent results might be due to difficulties in characterizing Tregs in large birth cohort studies; further studies are indispensable to evaluate the relevance of *in utero* events for cord blood Tregs and allergic predisposition.

In humans, Tregs are best characterized by the expression of the transcription factor FOXP3, which is essential for Treg induction and stability (8). However, FOXP3 is also expressed transiently in conventional CD4⁺ T cells upon activation (9–11). Recently, it has been shown that both induction and stabilization of FOXP3 expression is under epigenetic control (12–14). Sequence analyses revealed a highly conserved noncoding region in the *FOXP3* locus, the Treg-specific demethylated region (TSDR). Demethylation of the TSDR corresponds with the stability of FOXP3 expression in CD4⁺CD25^{hi} Tregs, whereas activated conventional human T cells expressing FOXP3 only transiently show a fully methylated TSDR (12). Thus, the demethylation state of the TSDR in the *FOXP3* gene has been suggested to be a valuable biomarker which can reliably detect and quantify stable CD4⁺CD25^{hi} Tregs in adults as well as in cord blood in large field studies (12, 15, 16).

The objective of the present study was to identify prenatal factors influencing Treg numbers at birth and evaluate the relevance of cord blood Treg numbers for allergic outcomes of the child during the first year of life. Maternal atopy might influence the development of foetal Tregs either directly or via maternal immune status during pregnancy. Furthermore, environmental exposure during pregnancy might influence the maternal immune status or directly modulate Treg numbers at birth. We therefore hypothesize that maternal/paternal atopy, maternal immune status during pregnancy and/or environmental exposure may influence the Treg number at birth with a potential relevance for the allergy risk in infancy.

Methods

For more detailed information, please see the methods section of Appendix S1.

Study design

Six-hundred and twenty-two mother–child pairs were recruited within the prospective birth cohort study LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) from March 2006 until December 2008 in Leipzig, Germany. Blood samples were obtained during pregnancy (mother, 34th week of gestation), at birth (venous umbilical cord blood) and on the child's first birthday. Data on confounding variables, prenatal exposure, life style factors and children's disease outcomes were obtained from questionnaires filled in by the parents 4 weeks before birth and on the child's first birthday. The present investigation comprises of only study participants who have received a complete blood analysis at pregnancy/birth and at the age of one ($n = 346$, greatest common subset, see Fig. S1 and methods/study design of Appendix S1). Participation was

voluntary, and informed consent was given by the parents. This study was approved by the Ethics Committees of the University of Leipzig (file reference 046-2006).

Parental history of atopy, prenatal exposure and outcomes in the first year of life

Information on parental history of atopy and prenatal exposure was assessed by detailed questionnaires. To address atopy history, we included asthma, hay fever and atopic dermatitis ('Did you ever suffer from asthma, allergic rhinitis, atopic dermatitis?'), 'Did your partner ever suffer from asthma, allergic rhinitis, atopic dermatitis?'). Exposure variables such as smoking/exposure to ETS at home ('Did you or anybody else smoke inside your dwelling during the last twelve months?'), any renovation activities ('Did you paint, install new furniture or change your flooring either before conception, during first to 13th gestational week, 14th to 26th gestational week or 27th to 40th gestational week?'), the usage of chemicals ('How often did you use disinfectants or air freshener during pregnancy?'), and traffic ('How is the distance between your dwelling and a main street, lower than ten metres or above 150 metre?') were considered.

Information on children's allergic outcomes was obtained from questionnaires. We included physician-diagnosed atopic dermatitis ('Has a doctor diagnosed your child with atopic dermatitis in the last twelve months?') and cradle cap ('Has a doctor diagnosed your child with cradle cap in the last twelve months?'). Furthermore, symptoms of atopic dermatitis observed by the parents were assessed ('Did your child suffer from any symptoms of atopic dermatitis in the last twelve months?').

Whole blood assay

Blood samples from mother–child pairs were processed within 6 h for further analysis. For cytokine analyses, whole blood samples were stimulated *ex-vivo* with phytohemagglutinin (PHA, 50 µg/ml; Sigma Aldrich, Munich, Germany) or lipopolysaccharide (LPS, 1 µg/ml, *E. coli* 026:B6; Sigma Aldrich). After incubating for 4 h at 37°C, samples were diluted with RPMI-1640 medium without supplements in a ratio of 1 : 1 and centrifuged. Cell-free supernatants were collected and stored at –80°C until subsequent analysis.

Cytokine measurement

Concentrations of IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, MCP-1 (CCL2), TNF- α and IFN- γ in the supernatants of stimulated whole blood samples were detected by flow cytometry using the BD CBA Human Soluble Flex Set system (BD Bioscience, Heidelberg, Germany) according to the manufacturer's instructions and as described previously (17). Detection limits for each cytokine were 3 pg/ml, excepting IL-5, where the detection limit was 2 pg/ml. IL-17A and IL-17E concentrations were measured by ELISA according to the manufacturer's instructions (eBiosciences, Frankfurt, Germany and ANTIGENIX AMERICA Inc, New York,

NY, USA, respectively). Detection limit was 4 pg/ml for IL-17A and 7 pg/ml for IL-17E. Cytokine concentrations below the detection limit were assigned a value that was half of the detection limit and were included in the analysis.

Immunoglobulin E measurement

The concentrations of total IgE and specific IgE against food (Fx5) and inhalant allergens (Sx1) in sera of 1-year-old children were determined by the Phadia CAP System (Phadia GmbH, Freiburg, Germany). The allergen multipanel Fx5 consists of hen's egg, cow's milk, wheat, fish, peanut and soy. Sx1 includes timothy, rye, mugwort, birch, house dust mite (*Dermatophagoides pteronyssinus*), cat and dog. Samples with a specific IgE concentration >0.35 kU/l and with total IgE concentration >3.8 kU/l were regarded as positive, according to published cut off values for 1-year-old children (18).

Isolation of DNA

Genomic DNA was isolated from whole blood using the DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, whole blood was treated with 20 µl Proteinase K (activity: 600 mAU/ml solution; Qiagen) and incubated with Buffer AL in a ratio of 1 : 1 (10 min, 56°C, 350 rpm).

Cell lysis was stopped by adding 200 µl of ethanol. The complete sample volume was transferred onto a QIAamp Mini spin column and centrifuged (17 540 g). DNA bound to the QIAamp membrane was washed two times using buffers AW1 and AW2 (17 540 g, 1 min; 20 590 g, 5 min). DNA was eluted by adding buffer AE and stored at 4°C until subsequent analysis.

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

Bisulfite treatment of genomic DNA was conducted using EpiTect 96 Bisulfite Kit (Qiagen), as described by the manufacturer. In brief, 1 µg of genomic DNA, 85 µl of the Bisulfite Mix and 35 µl of the DNA Protect Buffer were mixed in a 96-well EpiTect Conversion Plate, and bisulfite conversion was performed in a thermal cycler according to the manufacturers' instructions. Converted DNA was stored at -20°C until further analysis.

Quantification of demethylation in TSDR was performed by real-time PCR by Epiontis (Berlin, Germany; for details see Appendix S1). As described previously, the number of Tregs in cord blood is presented as percentage corresponding to the measured amount of TSDR demethylation in the *FOXP3* gene (15, 16).

Statistical analysis

Statistical tests were performed using Statistica for Windows Version 8.0 [StatSoft Inc. (Europe), Hamburg, Germany]. The chi-squared test for cross relationship was performed to ensure the equal distribution of parameters in the analysed

subcohort and the entire LINA cohort. Values for Treg numbers and cytokine concentrations were not normally distributed, implying the usage of nonparametric tests. First, bivariate analyses for the relationship between gender, parental atopy (defined as occurrence of asthma or atopic dermatitis or hay fever), environmental exposure during pregnancy and Treg numbers at birth (Mann-Whitney *U*-test) as well as between maternal cytokine production during pregnancy and cord blood Treg numbers were performed. Second, these relationships were verified using linear regression models with log-transformed Treg values adjusted for every single factor found to be significantly correlated with Treg numbers (calculation of adjusted mean ratios, adj MR). Each exposure variable in pregnancy was first tested individually as an influencing variable with children's cord blood Treg numbers being the outcome variable. In addition, we also tested all factors possibly influencing cord blood Treg numbers together in one regression model. Third, adjusted odds ratios (OR) were calculated for the relationship between cord blood Treg numbers and allergic outcomes in the first year of life. According to the results of bivariate analysis, either maternal atopic dermatitis or parental history of atopy was selected to confound this relationship. Although we did not observe a bivariate relationship between smoking/ETS exposure, gender, parental educational level and allergic outcomes, we decided to include these confounding variables because of their already described impact on allergy risk. Regarding atopic outcomes, analyses were comprised of the occurrence of atopic dermatitis and cradle cap, if diagnosed by a physician during the first year of life. Furthermore, the questionnaire reported symptoms of atopic dermatitis were considered. All *P* values <0.05 were considered to be significant. Due to the fact that we investigated *a priori* hypotheses, adjustment for multiple testing (Bonferroni correction) was not performed (19, 20).

Results

Characteristics of the study population

Complete data, including methylation analysis of the *FOXP3* locus in cord blood, maternal cytokine production during pregnancy and IgE measurements in children's blood samples that were 1 year of age, were available from 346 mother-child pairs (see Fig. S1; results, Appendix S1). Characteristics of the study population are listed in Table 1. There were no differences in the distribution of considered parameters in the analysed subcohort compared with the entire LINA cohort (Table 1). It is noteworthy that 64.7% of parents had a history of atopy, and 10.4% of children suffered from atopic dermatitis during the first year of life.

Relationship between gender, parental history of atopy and Treg numbers in cord blood

In the analysed subcohort, the median value of Treg numbers in cord blood, as detected by changes in demethylation of TSDR, was 1.18% [interquartile range (IQR) 0.73–1.71].

Table 1 Characteristics of the analysed subcohort and the entire LINA cohort during pregnancy and at birth ($n = 622$ mother–child pairs, seven twins) and at the age of one ($n = 606$)

	Analysed subcohort Pregnancy and birth	Entire LINA cohort	
Parameters	<i>n</i> (%), <i>N</i> = 346	<i>n</i> (%), <i>N</i> = 622	<i>P</i> value*
Gender of the child			
Male	189 (55.0)	327 (52.0)	0.671
Female	157 (45.0)	302 (48.0)	
Parental history of atopy [†]			
Negative	122 (35.3)	210 (33.8)	0.960
Single positive	155 (44.8)	291 (46.8)	
Double positive	69 (19.9)	121 (19.4)	
Maternal history of atopy [†]			
No	195 (56.4)	330 (53.1)	0.639
Yes	151 (43.6)	292 (46.9)	
Paternal history of atopy [†]			
No	204 (58.9)	381 (61.3)	0.729
Yes	142 (41.1)	241 (38.7)	
Parental education [‡]			
Low	7 (2.0)	17 (2.7)	0.947
Intermediate	82 (23.7)	144 (23.2)	
High	257 (74.3)	461 (74.1)	
Pet keeping during pregnancy			
None	199 (57.5)	358 (88.0)	0.376
Cat	64 (18.5)	113 (17.9)	
Dog	43 (12.4)	70 (11.1)	
Other	87 (25.1)	165 (26.2)	
Smoking inside dwelling during pregnancy			
Never	303 (87.6)	527 (84.7)	0.949
Occasionally	19 (5.5)	43 (6.9)	
Once per week	2 (0.6)	4 (0.7)	
Daily	22 (6.3)	48 (7.7)	
	At the age of 1 year		
	<i>n</i> (%), <i>N</i> = 346	<i>n</i> (%), <i>N</i> = 606	
Total & specific IgE at age one			
Enhanced total IgE (>3.8kU/l) [§]	274 (79.2)	404 (66.6)	0.988
Sensitization to food allergens (fx5) [¶]	52 (15.0)	79 (13.0)	
Sensitization to inhalant allergens (sx1) [¶]	13 (3.7)	17 (2.8)	
Atopic diseases at age one			
Symptoms of atopic dermatitis	38 (10.9)	59 (9.73)	0.970
Physician diagnosed atopic dermatitis	36 (10.4)	60 (9.90)	
Physician diagnosed cradle cap	202 (58.4)	354 (58.4)	

**P* value from chi-squared test for cross relationship.

†History of atopy is defined as occurrence of asthma or atopic dermatitis or hay fever.

‡Low, 9 years of schooling or less 'Hauptschulabschluss'; intermediate, 10 years of schooling 'Mittlere Reife'; high, 12 years of schooling or more '(Fach-)hochschulreife'.

§Values were categorized according to the published cut-off values (18). Concentrations >3.8 kU/l were regarded as enhanced.

¶According to the Pharmacia CAP System, concentrations >0.35 kU/l were regarded as positive.

In our analyses, the number of Tregs varied by gender of newborns. In cord blood of female neonates, the number of Tregs was significantly higher in comparison with the male ones ($P < 0.001$, Table 2). Furthermore, parental history of atopy was related to lower Treg numbers in cord blood (Table 2). Adjusted mean ratios for gender, maternal smoking/exposure to ETS at home and parental educational level

revealed an inverse relationship between parental history of atopy and Treg numbers in cord blood (adjusted MR = 0.78, 95% CI = 0.61–0.99 double positive, Fig. 1 and Table S1). Furthermore, we found lower Treg numbers in cord blood of neonates in the case of maternal hay fever (adjusted MR = 0.81, 95% CI = 0.68–0.97) and paternal asthma (adjusted MR = 0.60, 95% CI = 0.45–0.81) (Fig. 1, Table S1).

Table 2 Relationship between gender, parental history of atopy and Treg numbers in cord blood as detected by demethylation of Treg-specific demethylated region in the *FOXP3* gene

Parameter	Treg numbers in cord blood (%)		
	N	Median (IQR)	P value*
<i>Gender</i>			
Male	189	0.99 (0.61–1.55)	<0.001
Female	157	1.39 (0.86–1.95)	
<i>Parental history of atopy†</i>			
Negative	122	1.31 (0.83–1.86)	0.167
Single positive	155	1.14 (0.70–1.72)	
Double positive	69	1.04 (0.58–1.50)	
<i>Maternal history of atopy‡</i>			
Negative	195	1.21 (0.97–1.80)	0.264
Positive	151	1.13 (0.67–1.64)	
<i>Asthma</i>			
No	311	1.18 (0.74–1.72)	0.820
Yes	34	1.25 (0.63–1.60)	
<i>Hay fever</i>			
No	231	1.22 (0.80–1.80)	0.064
Yes	113	1.04 (0.58–1.59)	
<i>Atopic dermatitis</i>			
No	287	1.20 (0.73–1.71)	0.943
Yes	57	1.13 (0.80–1.72)	
<i>Paternal history of atopy‡</i>			
Negative	204	1.25 (0.77–1.83)	0.061
Positive	142	1.05 (0.58–1.59)	
<i>Asthma</i>			
No	299	1.25 (0.76–1.86)	<0.001
Yes	33	0.85 (0.44–1.38)	
<i>Hay fever</i>			
No	211	1.22 (0.77–1.78)	0.174
Yes	116	1.05 (0.59–1.70)	
<i>Atopic dermatitis</i>			
No	296	1.19 (0.75–1.82)	0.145
Yes	30	1.00 (0.51–1.51)	

Treg numbers are presented as medians and interquartile range (IQR).
 *Analyses were performed using Mann–Whitney *U*-test. Significant values are in boldface ($P < 0.05$).

†History of atopy is defined as occurrence of asthma or atopic dermatitis or hay fever.

Relationship between environmental exposure during pregnancy and Treg numbers in cord blood

Analysis of environmental exposure included maternal smoking/ETS at home, renovation activities, the usage of chemicals like disinfectants or air fresheners, as well as traffic exposure during pregnancy. Prenatal maternal smoking/ETS exposure at home as well as the usage of disinfectants during pregnancy were found to be associated with reduced Treg numbers in cord blood ($P = 0.039$, $P = 0.042$, respectively, Table 3). Treg numbers in cord blood did not vary significantly following renovation activities, usage of air fresheners, or traffic exposure during pregnancy (Table 3). After adjustment for gender, parental history of atopy, parental educational level and

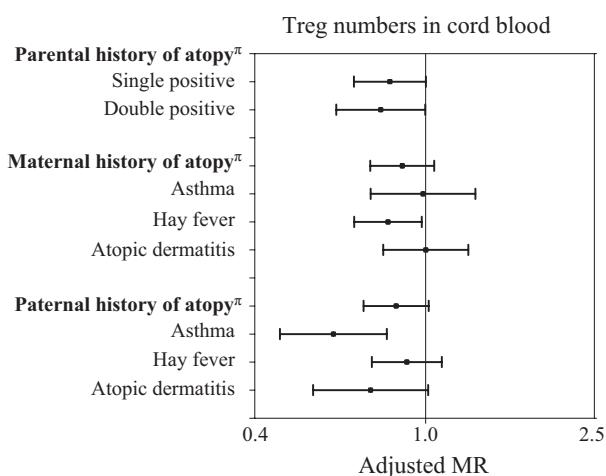


Figure 1 Relationship between parental history of atopy and Treg numbers in cord blood as detected by demethylation of Treg-specific demethylated region in the *FOXP3* gene. Linear regression models (shown are MR and 95% confidence intervals) were adjusted for gender, maternal smoking/environmental tobacco smoke exposure at home and parental educational level. ‡History of atopy is defined as occurrence of asthma or atopic dermatitis or hay fever.

Table 3 Association between exposures during pregnancy and Treg numbers in cord blood as detected by demethylation of Treg-specific demethylated region in the *FOXP3* gene

Exposure during pregnancy	Treg numbers in cord blood (%)		
	N	Median (IQR)	P value*
<i>Maternal smoking/ETS exposure at home</i>			
Never	303	1.17 (0.73–1.76)	0.039
Daily	22	0.85 (0.52–1.38)	
<i>Any renovation†</i>			
No	76	1.04 (0.62–1.72)	0.527
Yes	245	1.19 (0.73–1.66)	
<i>Usage of disinfectants</i>			
No	121	1.38 (0.81–1.80)	0.042
Yes	219	1.05 (0.70–1.68)	
<i>Usage of air freshener</i>			
No	137	1.35 (0.75–1.81)	0.135
Yes	205	1.05 (0.73–1.63)	
<i>Traffic‡</i>			
≤10m	39	1.32 (0.84–1.65)	0.713
>150m	119	1.20 (0.70–1.74)	

Treg numbers are presented as medians and interquartile range (IQR).

ETS, environmental tobacco smoke.

*Analyses were performed using Mann–Whitney *U*-test. Significant values are in boldface ($P < 0.05$).

†Questionnaire reported painting, installation of new furniture or changing the flooring either before conception or during pregnancy.

‡Values are presented in metre and refer to the distance between dwelling and main streets.

maternal cytokine production, only the association between maternal smoking/ETS exposure at home remained significant (adjusted MR = 0.89, 95% CI = 0.79–1.00, $P = 0.047$; Fig. 2, Table S2).

Relationship between maternal cytokine production during pregnancy and Treg numbers in cord blood

The relationship between maternal production of inflammatory markers IL-6, IL-8, IL-10, TNF- α and MCP-1 (CCL2) upon LPS stimulation as well as between PHA-induced production of maternal Th1/Th2/Th17 cytokines (IFN- γ , IL-4, IL-5, IL-13, IL-17E and IL-17A) and Treg numbers in cord blood was determined by linear regression adjusted for gender, parental history of atopy, maternal smoking/ETS at home and parental educational level (Fig. 3, Table S3). According to these models, maternal PHA-induced IL-13 (adjusted MR = 0.85, 95% CI = 0.75–0.96), IL-17E (adjusted MR = 0.92, 95% CI = 0.87–0.97) and IFN- γ (adjusted MR = 0.90, 95% CI = 0.83–0.98) production were associated with significantly reduced Treg numbers in cord blood (Fig. 3, Tables S3 and S4).

To verify the results obtained by testing possible influencing factors in separate models, we applied an additional modelling approach in which all factors potentially influencing Treg numbers at birth were included in one regression model. Following this approach, we obtained comparable results, confirming the data that was gained from single models (see Appendix S1, results and Fig. S2).

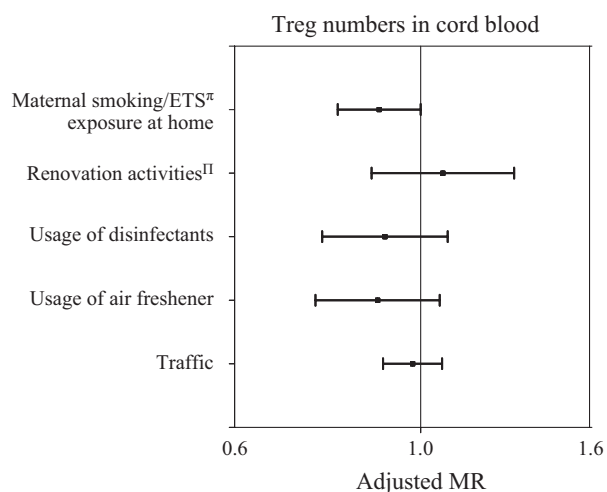


Figure 2 Relationship between maternal prenatal exposure and Treg numbers in cord blood as detected by demethylation of Treg-specific demethylated region in the *FOXP3* gene. Linear regression models (shown are MR and 95% confidence intervals) were adjusted for gender, parental history of atopy, parental education and maternal production of IL-13, IL-17E, IFN- γ upon phytohemagglutinin stimulation. ^aETS, environmental tobacco smoke. ^bQuestionnaire reported painting, installation of new furniture or changing the flooring either before conception or during pregnancy.

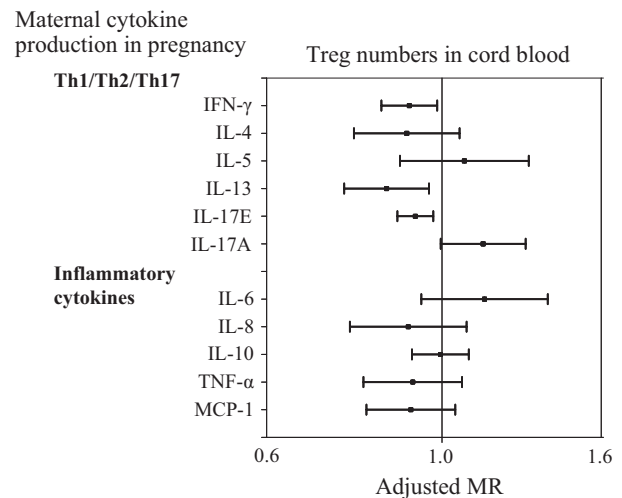


Figure 3 Relationship between phytohemagglutinin/lipopolysaccharide-stimulated cytokine production in maternal blood and Treg numbers in cord blood as detected by demethylation of Treg-specific demethylated region in the *FOXP3* gene. Linear regression models (shown are MR and 95% confidence intervals) were adjusted for gender, parental history of atopy, maternal smoking/environmental tobacco smoke exposure at home and parental educational level.

Factors influencing maternal cytokines

We analysed the relationship between maternal history of atopy, environmental exposure and cytokine production during pregnancy. Increased maternal IL-13 production was related to maternal history of atopy, maternal hay fever and asthma, whereas increased IL-17E levels were associated with the usage of disinfectants during pregnancy (see results Appendix S1, Tables S6 and S7).

Relationship between Treg numbers in cord blood, diseases and atopic sensitization in the first year of life

Analyses of the relationship between cord blood Treg numbers and allergic outcomes included the occurrence of physician-diagnosed cradle cap, atopic dermatitis (symptoms and physician diagnosed) as well as sensitization to food and inhalant allergens in the first year of life (Table 4, Table S5, results, Appendix S1).

According to our adjusted logistic regression models, children with lower Treg numbers in cord blood had a significantly higher risk of developing an atopic dermatitis in the first year of life (physician-diagnosed: adjusted OR = 1.55, 95% CI = 1.00–2.41, $P = 0.048$, Fig. 4, Table S5). Upon analysis of specific IgE levels in sera of the 1-year-old children, we found a higher risk for sensitization against food allergens in case of lower Treg numbers at birth (adjusted OR = 1.55, 95% CI = 1.06–2.25, $P = 0.020$, Fig. 4, Table S5).

Discussion

Our results reveal differences in cord blood Treg numbers stably expressing *FOXP3* depending on gender, family history of

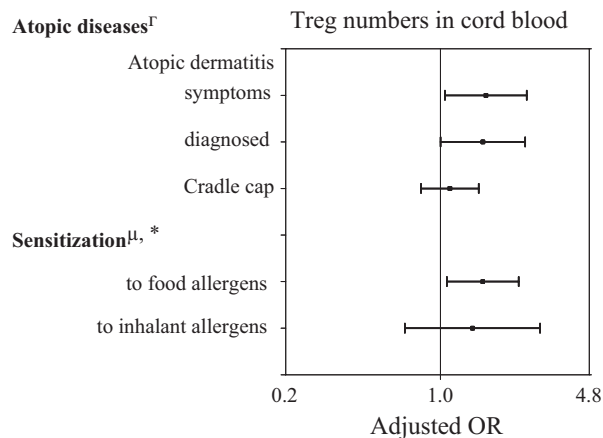


Figure 4 Relationship between Treg numbers, atopic diseases and sensitization in cord blood as detected by demethylation of Treg-specific demethylated region in the *FOXP3* gene. [†]Logistic regression models (OR and 95% confidence intervals) were adjusted for gender, maternal atopic dermatitis, maternal smoking/environmental tobacco smoke exposure (ETS) at home and parental educational level. [‡]Adjusted odds ratios for gender, parental history of atopy and parental educational level (because of the distribution of cases maternal smoking/ETS exposure at home could not be considered). *According to the Pharmacia CAP System, concentrations >0.35 kU/l were regarded as positive.

atopy as well as prenatal exposure to tobacco smoke. In our study, we found that being male or having parents with a history of atopy was related to lower Treg numbers in cord blood. Furthermore, the capacity of maternal blood cells to produce cytokines (IFN- γ , IL-13 and IL-17E) upon stimulation was inversely related to Treg numbers in cord blood. In addition, maternal smoking/exposure to ETS at home was associated with decreased Treg numbers. Finally, the results indicate that children with low Treg numbers at birth might

Table 4 Association between Treg numbers in cord blood as detected by demethylation of Treg-specific demethylated region in the *FOXP3* gene and allergic diseases as well as sensitization to food and inhalant specific allergens in the first year of life

	Treg numbers in cord blood (%)		
Outcome at age one	<i>N</i>	Median (IQR)	<i>P</i> value*
<i>Atopic diseases</i>			
Atopic dermatitis			
Symptoms			
No	307	1.22 (0.75–1.74)	0.021
Yes	38	0.86 (0.51–1.47)	
Diagnosed			
No	309	1.22 (0.75–1.72)	0.046
Yes	36	0.91 (0.54–1.39)	
Cradle cap			
No	143	1.28 (0.74–1.72)	0.535
Yes	202	1.15 (0.73–1.69)	
<i>Sensitization*</i>			
To food allergens			
No	294	1.21 (0.76–1.76)	0.032
Yes	52	0.97 (0.76–1.76)	
To inhalant allergens			
No	333	1.19 (0.74–1.70)	0.549
Yes	13	1.04 (0.55–1.71)	

Treg numbers in cord blood are presented as medians and inter-quartile range (IQR).

*Analyses were performed using Mann–Whitney *U*-test. Significant values are in boldface ($P < 0.05$).

[†]According to the Pharmacia CAP System, concentrations >0.35 kU/l were regarded as positive.

be at a higher risk of developing atopic dermatitis or sensitization to food allergens in the first year of life (Fig. 5).

The strength of our study lies in the fact that the measurement of Treg numbers was performed by analysing the

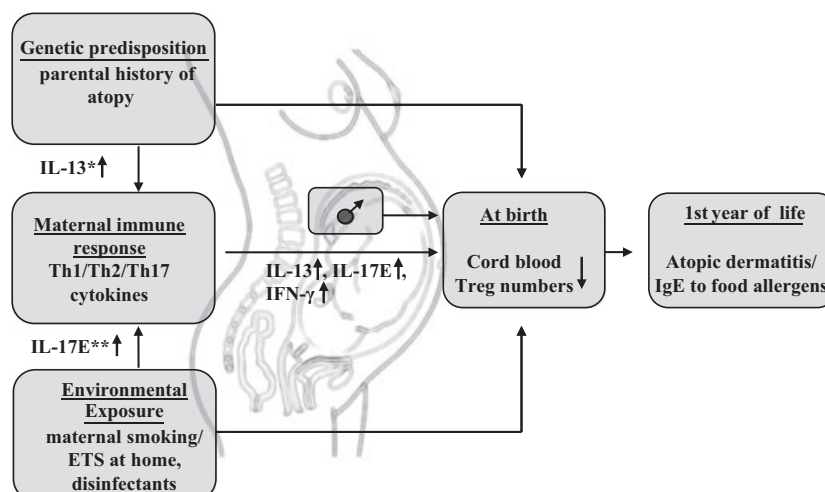


Figure 5 Overview on prenatal factors that might be associated with alterations in Treg numbers and thereby modulate the susceptibility to diseases at the age of one. *Maternal atopy, asthma or hay fever; **usage of disinfectants during pregnancy.

methylation state of the TSDR in the *FOXP3* gene, which constitutes as an exclusive, specific and reliable marker for stable Tregs (12–15). We analysed *TSDR* demethylation in relation to various factors in the prenatal environment, set in a large prospective birth cohort. Our study comprises mother–child pairs that are well characterized regarding immune parameters as well as for atopic outcomes and exposure variables. A weakness of the LINA study in general is the potential bias by high rates of participating atopic parents (64.7%). We have considered this point by including parental atopy history as confounding variable in the regression models. One other limitation of the study is the low number of exposed participants to certain variables (e.g. daily smoking/ETS at home $n = 22$). We are aware of the fact that testing the possible influencing factors on Treg numbers in separate models might not be the most convincing approach. To overcome this weakness, we additionally tested all potentially influencing factors in one regression model. Both approaches generated similar results. As usual in population-based studies, we also have the limitation to define the cause–effect relation in our observed associations.

In our study cohort, the median value of Treg numbers in cord blood, as detected by changes in demethylation of TSDR, was 1.18% (IQR = 0.73–1.71). This value is comparable to data from a recent publication by Schaub et al. (21) using the same method for Treg quantification. Depending on farm exposure, they found median Treg frequencies to be between 1.6–2.0 (IQR = 1.2–2.4).

Results from epidemiologic studies addressing the influence of parental history of atopy on cord blood Tregs are conflicting. Our finding that a family history of atopy is associated with reduced Treg numbers in cord blood is in line with previous studies demonstrating an impairment of Tregs at birth in neonates with a hereditary allergy risk (5, 6, 22). In contrast, Rindsjö et al. (7) did not detect an effect of maternal allergic disease on Treg subsets in cord blood. This might be explained by differences in the experimental setup. In the study of Rindsjö et al., Tregs were analysed flow cytometrically by the expression of FoxP3 and a panel of T-cell homing markers, whereas in the other studies, the suppressive capacity of Tregs and/or the expression of Treg-related proteins including FoxP3 were determined (6, 22).

In our study, male neonates had significantly lower Treg numbers compared with females, suggesting gender-dependent differences in Treg development. By now, there is no evidence concerning the relationship between gender and Treg numbers. However, gender-associated disproportion has been described in immune development and allergic immune responses [reviewed in (23, 24)]. As Tregs play an important role in preventing allergic diseases, our results (lower Treg numbers in male cord blood) fit with the well-documented finding that atopic diseases in early childhood are predominant in boys (23, 25).

Concerning maternal intrinsic factors, it has been described previously that the maturation of the foetal immune system is determined by maternal immune status, atopic disease, as well as the capacity of adaptive and innate immune responses (1, 2). For example, maternal sensitization to allergens was

found to be associated with an elevated production of the Th2 cytokine IL-13 in human infants (26). In a previous study, we demonstrated that total maternal IgE in pregnancy was predictive for total IgE in cord blood and in the first year of life, and that maternal TNF- α and IL-10 production during pregnancy was an indicator for high concentrations of these cytokines in children's blood at the age of one (27). Furthermore, we showed that high blood TNF- α concentrations during pregnancy are protective against sensitization to inhalant allergens in 1-year-old children (27). There are no data in the literature concerning the influence of maternal immune responses in pregnancy on the development of foetal Tregs. In our study Th1, Th2 and Th2-related cytokines IFN- γ , IL-13 and IL-17E (IL-25) were associated with reduced Treg numbers in cord blood. Thus, a strong maternal adaptive immune response in pregnancy might antagonize the development of foetal Tregs. Our data therefore support previous *in vitro* findings demonstrating an inhibitory influence of Th1/Th2 polarizing cytokines on Treg development (28). We furthermore investigated factors influencing maternal cytokine production during pregnancy (see Appendix S1). Focusing on maternal cytokines relevant for children's Treg development, we found that increased maternal IL-13 production was related to maternal history of atopy, maternal hay fever and asthma, whereas increased IL-17E levels were associated with the usage of disinfectants during pregnancy. Thus, at least maternal atopy and usage of disinfectants might increase maternal IL-13 and IL-17E production and thereby influence the number of Treg in cord blood.

In our study, maternal smoking/exposure to ETS at home was associated with lower Treg numbers in cord blood compared with neonates without exposure. The influence of maternal smoking on Tregs at birth has not been investigated so far. However, maternal smoking has been shown to modify neonatal immune functions. This results in higher neonatal Th2 responses, as well as perturbed toll-like receptor (TLR)-mediated responses in cord blood at birth (29, 30). Furthermore, TLR-mediated responses at birth have been linked to the expression of Treg-related genes (22).

In addition to maternal intrinsic factors and exposure, paternal genetic/epigenetic factors may also affect the maturation of the foetal immune system. Analysing the influence of genetic predisposition on Tregs as detected by changes in the demethylation of TSDR in our study depicted a significant relationship between paternal asthma and lower Treg numbers in cord blood. Thus, in case of inheritance of *FOXP3* methylation patterns, the atopic father seems to play an important role, too. Considering the fact that boys had reduced Treg numbers, male gender might in general be a predisposition in terms of lower Treg numbers determined by increased methylation of TSDR in the *FOXP3* gene.

Epigenetic alterations are discussed as one potential mechanism mediating the impact of the environment on gene expression (3). Thus, environmental factors might have the potential to influence the epigenetic fixation of the Treg phenotype by TSDR demethylation or methylation. Recently, it has been shown that prenatal tobacco smoke exposure affects global and gene-specific DNA methylation (31). The authors

demonstrated that *in utero* exposure to tobacco smoke is related to global hypomethylation, whereas single CpG loci were hypermethylated in several genes. Whether tobacco smoke exposure during pregnancy directly influences epigenetic regulation of *FOXP3* or involves other pathways remains to be elucidated.

Finally, we were interested in the predictive value of low Treg numbers in cord blood regarding the development of diseases and allergic sensitization in the first year of life. Even though Tregs in humans constitute only up to two per cent of the total peripheral CD4⁺ T cells (32), we were able to detect significant associations between cord blood Treg numbers and allergic outcomes. We demonstrated that atopic dermatitis and sensitization to food allergens in the first year of life were associated with lower Treg numbers in cord blood. Our findings are in line with results from a study demonstrating an association between impaired suppressive function of Tregs in cord blood at birth and egg allergy in the first year of life (33). Thus, Treg numbers in cord blood may be considered as a predictive marker for the development of allergic diseases later in life.

In a previous investigation of the LINA study, we found that maternal cytokines during pregnancy did not directly correlate with atopic dermatitis of the child at the age of one (27). However, we found an association between maternal and children's immune response. Deduced from the results of the present study, we hypothesized that maternal cytokines in pregnancy do not directly influence the development of atopic diseases in the offspring, but rather they might affect the maturation of the foetal immune system (e.g. Treg development) and thereby the development of allergies and allergic sensitization.

Altogether our results suggest that male sex, maternal intrinsic factors (atopy history, IL-13 and IL-17E production), parental history of atopy as well as ETS exposure during pregnancy are related to lower Treg numbers in cord blood (Fig. 5). As we found a relationship between maternal history of atopy and maternal IL-13 production, we hypothesize that the maternal atopic background affects foetal Treg development via the cytokine milieu during pregnancy. Prenatal tobacco smoke exposure seems to affect foetal Tregs directly without affecting maternal immune responses, which might be relevant for Treg development. As a consequence of lower cord blood Treg numbers, the risk for the development of allergic sensitization against food allergens and atopic dermatitis increases.

Mechanisms by which Treg affects the allergic outcome and how environmental factors act on Treg development have to be elucidated in future studies. Nevertheless, by measuring demethylation of TSDR in the *FOXP3* gene, the results of our study provide evidence for prenatal modulation of Treg numbers and encourage further investigation in this direction.

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Authors' contribution

Each named author substantially contributed to this paper. Gunda Herberth, Mario Bauer, Stefan Röder, Michael Borte, and Irina Lehmann were involved in the development of the study design and the field work. Denise Hinz, Gunda Herberth, Mario Bauer and Sven Olek performed the *FOXP3* TSDR demethylation measurement. Stefan Röder, Denise Hinz, Gunda Herberth and Irina Lehmann contributed to the statistical analysis. Ulrich Sack contributed with IgE measurements and discussion of the IgE data. Jochen Huehn and Jan Christoph Simon contributed with the discussion of *FOXP3* TSDR demethylation and allergy outcome. Gunda Herberth, Denise Hinz and Irina Lehmann wrote the paper; all authors were involved in the revision of the final text.

Conflict of interest

All authors have declared that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Flow chart of the LINA study population with the analysed subcohort.

Figure S2. Regression model with all factors possibly influencing cord blood Treg numbers.

Table S1. Relationship between parental history of atopy and Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.

Table S2. Relationship between maternal prenatal exposure and Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.

Table S3. Relationship between PHA-/LPS-stimulated cytokine production in maternal blood during pregnancy and number of Tregs in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.

Table S4. Cytokine production during pregnancy in PHA-/LPS-stimulated blood samples. Values are presented as medians and the interquartile range.

Table S5. Relationship between Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene and the development of allergy as well as sensitisation to food or inhalant specific allergens in the first year of life.

Table S6. (a) Association between maternal history of atopy and PHA induced maternal cytokine production in pregnancy. Cytokine concentrations are presented as medians and interquartile range (IQR). (b) Association between maternal history of atopy and PHA induced maternal cytokine production in pregnancy. Cytokine concentrations are presented as medians and interquartile range (IQR).

Table S7. (a) Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR). (b) Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR). (c) Association between exposures during pregnancy and maternal cytokine

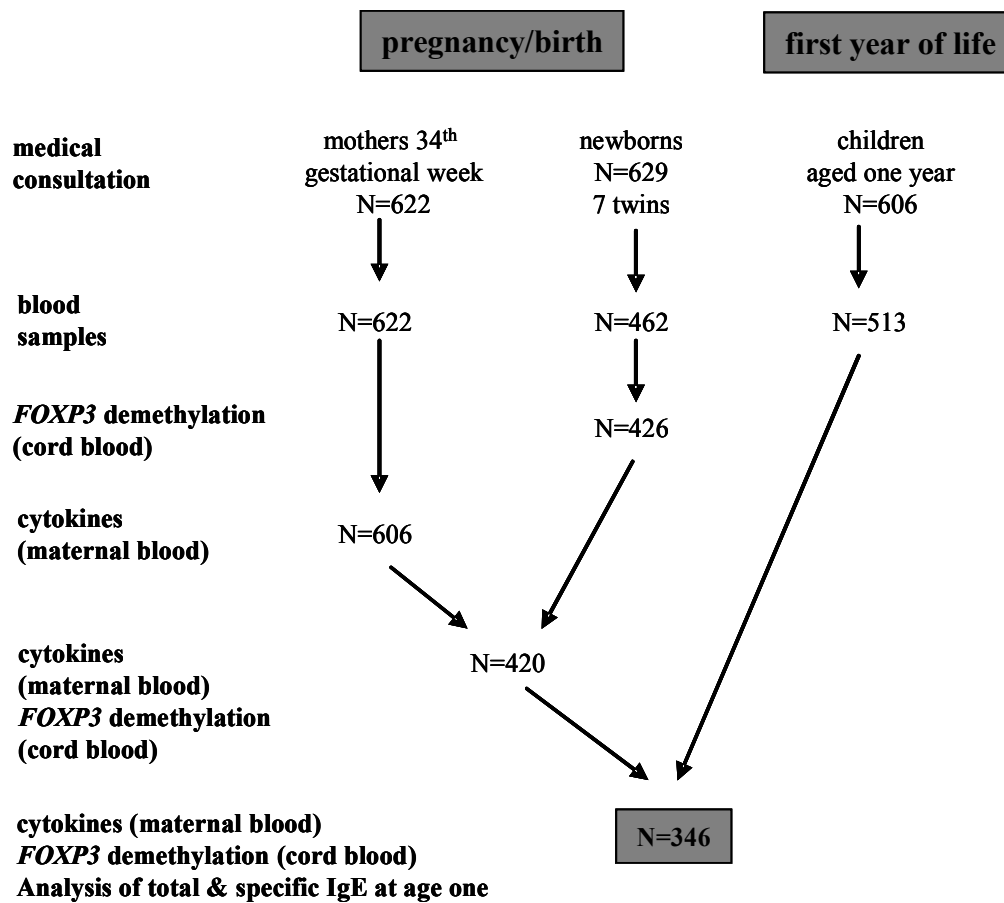
production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR).

Appendix S1. Methods and Results.

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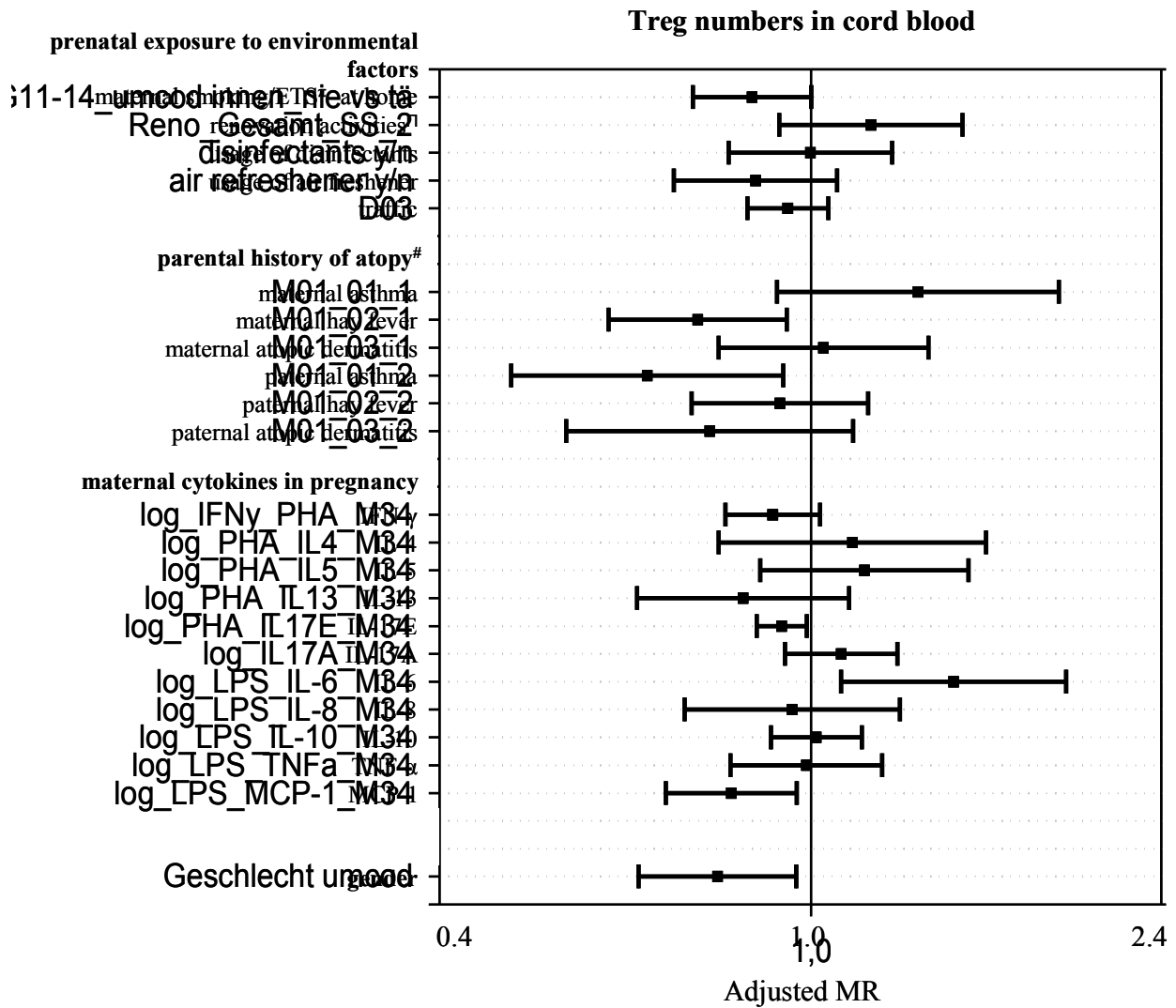
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Supplementary Fig. S1. Flow chart of the LINA study population with the analysed subcohort.

Relationship between prenatal factors and Treg numbers in cord blood



Supplementary Table S1. Relationship between parental history of atopy and Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.[#]

Parental history of atopy vs. Treg numbers in cord blood				
	Crude MR	<i>P</i> value	Adjusted MR*	<i>P</i> value ^π
Parental history of atopy^μ				
single positive	0.88 (0.73-1.07)	0.201	0.82 (0.68-1.00)	0.048
double positive	0.82 (0.65-1.03)	0.084	0.78 (0.61-0.99)	0.045
Mother				
history of atopy ^μ	0.92 (0.78-1.08)	0.322	0.88 (0.74-1.04)	0.144
asthma	0.98 (0.74-1.30)	0.907	0.98 (0.74-1.30)	0.922
hay fever	0.83 (0.70-1.00)	0.047	0.81 (0.68-0.97)	0.026
atopic dermatitis	1.04 (0.83-1.30)	0.696	1.00 (0.79-1.25)	0.996
Father				
history of atopy ^μ	0.87 (0.73-1.03)	0.102	0.85 (0.71-1.01)	0.071
asthma	0.61 (0.46-0.81)	<0.001	0.60 (0.45-0.81)	<0.001
hay fever	0.89 (0.74-1.07)	0.226	0.90 (0.74-1.09)	0.282
atopic dermatitis	0.80 (0.59-1.08)	0.140	0.74 (0.54-1.01)	0.057

MR, mean ratio

[#]Analysis were performed using a linear regression model with log transformed data

*Adjusted mean ratios for gender, maternal smoking/ETS exposure at home and parental educational level

^πValues in boldface are significant (*P* < 0.05)

^μHistory of atopy is defined as: occurrence of asthma or atopic dermatitis or hay fever.

Supplementary Table S2. Relationship between maternal prenatal exposure and Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.[#]

maternal prenatal exposure vs. Treg numbers in cord blood				
	Crude MR	<i>P</i> value	Adjusted MR*	<i>P</i> value ^π
Maternal smoking/ETS exposure at home	0.92 (0.82-1.03)	0.163	0.89 (0.79-1.00)	0.047
Renovation activities [‡]	1.05 (0.86-1.29)	0.578	1.06 (0.87-1.29)	0.533
Usage of disinfectants	0.86 (0.73-1.03)	0.109	0.90 (0.76-1.07)	0.257
Usage of air freshener	0.90 (0.76-1.07)	0.237	0.88 (0.74-1.05)	0.167
Traffic	0.98 (0.90-1.06)	0.686	0.97 (0.90-1.06)	0.587

MR. mean ratio

[#]Analysis were performed using a linear regression model with log transformed data

*Adjusted mean ratios for gender, parental history of atopy, parental educational level and maternal production of IL-13 IL-17E, IFN-γ upon PHA stimulation

^πValues in boldface are significant ($P < 0.05$)

[‡]refers to the questionnaire reported painting, installation of new furniture or changing the flooring during the last twelve months before the birth of the child

Supplementary Table S3. Relationship between PHA-/LPS-stimulated cytokine production in maternal blood during pregnancy and number of Tregs in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.[#]

Cytokine production in maternal blood samples (34 th gest. week)	cytokine production in maternal blood vs. Treg numbers in cord blood			
	Crude MR	<i>P</i> value	Adjusted MR*	<i>P</i> value ^π
Th1/Th2/Th17				
IFN-γ	0.91 (0.85-0.98)	0.024	0.90 (0.83-0.98)	0.020
IL-4	0.88 (0.76-1.02)	0.102	0.90 (0.77-1.05)	0.183
IL-5	1.12 (0.93-1.34)	0.211	1.06 (0.88-1.28)	0.486
IL-13	0.83 (0.74-0.94)	0.004	0.85 (0.75-0.96)	0.009
IL-17E	0.92 (0.87-0.97)	0.002	0.92 (0.87-0.97)	0.003
IL-17A	1.09 (0.97-1.23)	0.132	1.12 (0.99-1.27)	0.053
Inflammatory cytokines				
IL-6	1.14 (0.95-1.37)	0.125	1.13 (0.94-1.36)	0.178
IL-8	0.91 (0.77-1.08)	0.305	0.90 (0.76-1.07)	0.249
IL-10	1.01 (0.93-1.09)	0.774	0.99 (0.91-1.08)	0.912
TNF-α	0.90 (0.78-1.03)	0.143	0.91 (0.79-1.06)	0.238
MCP-1 (CCL2)	0.94 (0.83-1.04)	0.386	0.91 (0.80-1.03)	0.163

MR, mean ratio

[#]Analysis were performed using a linear regression model with log transformed data

*Adjusted mean ratios for gender, parental history of atopy, maternal smoking/ETS exposure at home and parental educational level

^πValues in boldface are significant (*P* < 0.05)

Supplementary Table S4. Cytokine production during pregnancy in PHA-/LPS-stimulated blood samples. Values are presented as medians and the interquartile range.

Maternal cytokine production (34 th gest. week)	Concentration in pg/ml Median (IQR)
Th1/Th2/Th17	
IFN-γ	407.60 (233.36-806.36)
IL-4	15.17 (10.80-20.94)
IL-5	3.29 (2.20-4.01)
IL-13	21.46 (14.45-32.37)
IL-17E	105.48 (35.80-265.33)
IL-17A	82.40 (51.98-125.23)
Inflammatory cytokines	
IL-6	9978.35 (7247.99-13142.36)
IL-8	2945.32 (2247.59-4373.74)
IL-10	12.43 (6.33-24.83)
TNF-α	1883.12 (1319.18-2896.18)
MCP-1 (CCL2)	395.95 (268.74-594.26)

Supplementary Table S5. Relationship between Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene and the development of allergy as well as sensitisation to food or inhalant specific allergens in the first year of life.[#]

Outcome at age one	Treg numbers in cord blood			
	Crude OR (95% CI)	<i>P</i> value	Adjusted OR (95% CI)	<i>P</i> value
Atopic diseases*				
atopic dermatitis				
symptoms	1.53 (1.03-2.28)	0.034	1.60 (1.04-2.45)	0.028
diagnosed	1.42 (0.94-2.15)	0.087	1.55 (1.00-2.41)	0.048
cradle cap	1.07 (0.80-1.42)	0.616	1.10 (0.81-1.48)	0.518
Sensitisation^{‡, †}				
to food allergens	1.45 (1.01-2.07)	0.040	1.55 (1.06-2.25)	0.020
to inhalant allergens	1.23 (0.63-2.41)	0.526	1.39 (0.69-2.81)	0.351

[#]Analysis was performed using a logistic regression model with log transformed Treg values; values in boldface are significant ($P < 0.05$).

*Adjusted odd ratios for gender, maternal atopic dermatitis, maternal smoking/ETS exposure at home and parental educational level.

[‡] Adjusted odd ratios for gender, parental history of atopy and parental educational level. Due to the distribution of cases maternal smoking/ETS exposure could not be considered.

[†] According to the Pharmacia CAP System concentrations >0.35 kU/l were regarded as positive.

Supplementary Table S6a. Association between maternal history of atopy and PHA induced maternal cytokine production in pregnancy. Cytokine concentrations are presented as medians and interquartile range (IQR).[#]

Parameter	Cytokine production in the 34 th gest. week (pg/ml)			
	Maternal history of atopy [‡]		P value	Maternal history of atopy
	Median (IQR)			
	negative (195)	positive (151)		no (311)
IFN- γ	398.56 (213.46-839.24)	424.03 (257.28-757.01)	0.678	404.57 (220.73-806.36)
IL-4	14.74 (10.45-20.12)	15.74 (11.22-22.70)	0.067	15.00 (10.70-20.45)
IL-5	3.22 (2.14-3.93)	3.49 (2.35-4.08)	0.154	3.25 (2.14-3.97)
IL-13	19.59 (13.24-28.61)	25.24 (15.76-35.36)	0.005	20.48 (14.17-30.84)
IL-17E	90.93 (26.10-229.59)	109.78 (48.57-322.78)	0.249	104.97 (32.19-245.75)
IL-17A	80.05 (47.82-124.70)	83.91 (53.79-131.52)	0.334	83.03 (52.45-125.35)

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

[‡]History of atopy is defined as: occurrence of asthma or atopic dermatitis or hay fever.

Supplementary Table S6b. Association between maternal history of atopy and PHA induced maternal cytokine production in pregnancy. Cytokine concentrations are presented as medians and interquartile range (IQR).[#]

Parameter	Cytokine production in the 34 th gest. week (pg/ml)			
	Maternal hay fever	P value		Maternal history of atopy
	Median (IQR)			Median (IQR)
	no (231)	yes (113)		no (287)
IFN- γ	427.55 (213.46-838.06)	401.64 (257.28-750.49)	0.881	407.71 (217.77-812.19)
IL-4	14.86 (10.45-20.45)	15.68 (11.75-22.11)	0.101	15.00 (10.70-20.60)
IL-5	3.25 (2.10-4.06)	3.31 (2.49-3.98)	0.544	3.22 (2.14-3.93)
IL-13	20.41 (13.27-30.62)	24.93 (15.98-33.76)	0.044	20.67 (14.44-30.84)
IL-17E	91.88 (25.51-265.33)	109.78 (52.94-275.31)	0.343	101.85 (38.04-265.33)
IL-17A	78.67 (48.38-125.23)	88.02 (56.98-131.52)	0.226	82.64 (51.98-123.42)

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

Supplementary Table S7a. Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR). #

Parameter	Cytokine production in the 34 th gest. week (pg/ml)					
	maternal smoking/ ETS ^π exposure at home			Any renovation [¶]		
	<i>P</i> value			<i>P</i> value		
	Median (IQR)			Median (IQR)		
	never (303)	daily (22)		no (76)	yes (245)	
IFN-γ	424.04 (247.46-812.19)	351.14 (170.69-652.26)	0.212	410.81 (244.38-729.62)	435.66 (247.46-838.06)	0.702
IL-4	15.53 (10.79-21.26)	12.60 (8.45-20.12)	0.188	15.69 (10.28-24.12)	15.13 (10.87-20.45)	0.474
IL-5	3.29 (2.19-4.06)	3.18 (1.50-3.89)	0.413	3.36 (2.23-4.12)	3.22 (2.19-3.93)	0.303
IL-13	21.58 (14.94-31.93)	19.58 (11.95-32.42)	0.461	20.26 (15.22-34.73)	21.58 (13.31-31.49)	0.690
IL-17E	101.85 (40.04-275.31)	85.67 (15.86-244.11)	0.674	103.41 (54.92-270.32)	110.20 (40.04-275.33)	0.925
IL-17A	83.91 (54.22-127.05)	55.04 (36.75-86.65)	0.047	78.61 (49.91-130.87)	83.70 (52.47-122.56)	0.959

Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

^πETS; environmental tobacco smoke

[¶]refers to questionnaire reported painting, installation of new furniture or changing the flooring either before conception or during pregnancy

Supplementary Table S7b. Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR). #

Parameter	Cytokine production in the 34 th gest. week (pg/ml)					
	Usage of disinfectants		<i>P</i> value	Usage of air freshener		<i>P</i> value
	Median (IQR)			Median (IQR)		
	no (121)	yes (219)		no (137)	yes (205)	
IFN- γ	380.27 (205.97-841.79)	431.49 (249.84-803.98)	0.474	461.78 (208.28-852.22)	404.57 (249.84-762.82)	0.784
IL-4	14.59 (10.64-19.16)	15.65 (11.22-21.93)	0.128	15.85 (11.53-20.91)	14.80 (10.70-21.26)	0.403
IL-5	3.31 (2.27-4.00)	3.29 (2.19-4.02)	0.937	3.41 (2.19-4.08)	3.29 (2.27-3.93)	0.625
IL-13	19.50 (13.27-30.18)	22.91 (15.51-32.69)	0.050	21.11 (13.93-30.62)	21.66 (14.74-33.21)	0.761
IL-17E	86.89 (25.15-192.85)	112.15 (48.57-310.06)	0.036	125.34 (51.43-297.98)	88.64 (26.10-231.29)	0.233
IL-17A	84.60 (55.18-123.42)	80.25 (50.26-126.62)	0.598	86.38 (55.72-141.79)	75.64 (47.50-120.26)	0.050

#Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

Supplementary Table S7c. Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR). [#]

Parameter	Cytokine production in the 34 th gest. week (p	
	Traffic*	
	Median (IQR)	
	<10 m (39)	>=150 m (119)
IFN-γ	354.91 (242.69-864.72)	407.49 (237.99-872.49)
IL-4	13.33 (11.52-22.11)	15.67 (11.66-21.86)
IL-5	3.16 (1.50-3.84)	3.56 (2.35-4.12)
IL-13	21.97 (15.78-30.34)	22.65 (13.92-34.27)
IL-17E	126.44 (24.77-303.92)	91.88 (43.86-209.16)
IL-17A	73.32 (48.38-125.23)	91.26 (53.91-139.83)

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

*Values are presented in meter and refer to the distance between dwelling and main street.

Supporting information

Cord blood Tregs with stable *FOXP3* expression are influenced by prenatal environment and associated with atopic dermatitis at the age of one year

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Appendix S1

Methods

Real-time-PCR for quantification of the Treg-specific demethylated region (TSDR) in the FOXP3 gene

Real-time-PCR for quantification of the Treg-specific demethylated region (TSDR) in the *FOXP3* Gene was performed in 20 µl using Roche LightCycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany) containing 15 pmol of methylation or non-methylation specific forward and reverse primer for TSDR, 5 pmol hydrolysis probe, 200 ng lambda-DNA (New England Biolabs, Frankfurt, Germany) and 30 ng bisulfite-treated genomic DNA template or respective amount of plasmid standard (12). Each sample was analysed in triplicate (15, 16). These analyses were performed by Epiontis GmbH (Berlin, Germany).

Results

Study population

In supplementary Figure S1 we schematically depicted the number of samples at each stage/analysis ending with the greatest common subset of mother-child pairs at birth and children's samples at the age of one including only children with complete blood samples and analyses (n=346, greatest common subset).

Missing samples mainly came off because blood samples from mother-child pairs at birth (n=622 maternal, n=462 cord blood) do not fully match with blood samples from children at the age of one (n=513). The greatest common subset of mother-child pairs with blood samples at pregnancy/birth and with *FOXP3* demethylation measurements in children's samples was n=420. A number of n=513 blood samples were available at the age of one. All of them were analysed for specific IgE at the age of one and n=346 matched with the pregnancy/birth

samples having complete data for maternal cytokines and *FOXP3* demethylation in cord blood. Thus, this sub cohort did not result by a selection per se but by availability of blood samples. Due to missing data from questionnaires in some cases the final case numbers vary from 312 to 346.

Influencing factors on cord blood Treg numbers – results from a combined regression model

A linear regression model comprised of all factors with potential impact on Treg numbers at birth, revealed comparable results as described for the single models. The effects of gender, maternal smoking/ETS exposure, and parental history of atopy remained significant. Regarding the influence of maternal cytokines during pregnancy on Treg numbers, we found a stable effect for IL-17E, while IL-13 and IFN- γ lost significance in the combined model. Correlation analyses have shown that IL-17E, IL-13 and IFN- γ correlated with each other, raising the problem of multicollinearity. Therefore, the loss of IL-13 and IFN- γ in the combined model has to be interpreted with caution.

In addition to IL-17E, MCP-1 was found to be associated with lower Treg numbers in cord blood too, whereas IL-6 was positively correlated (Supplementary Figure S2). This result may point to an additional impact of the maternal innate immune response on foetal Treg development.

Relationship between Treg numbers in cord blood, diseases and atopic sensitisation in the first year of life

Analyses of the relationship between cord blood Treg numbers and allergic outcomes included the occurrence of physician diagnosed cradle cap, atopic dermatitis (symptoms and physician diagnosed), as well as sensitisation to food and inhalant allergens in the first year of

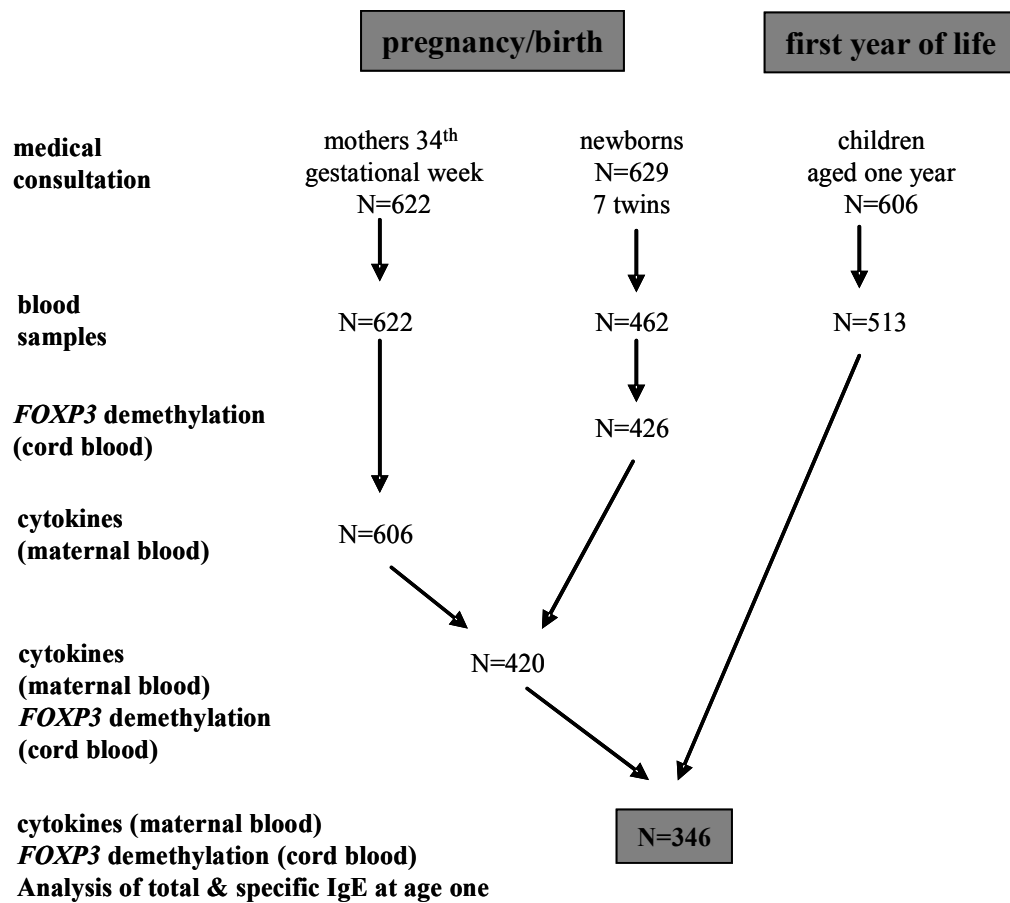
life. Sensitisation was regarded as positive in case of specific IgE values > 0.35 kU/l in children's sera, according to the Phadia CAP System. Children sensitised to food allergens had reduced Treg numbers compared to those that were not sensitised (median: 0.97 vs. 1.21, $P=0.032$, Table 4). Similarly, children with physician-diagnosed or with symptoms for atopic dermatitis had significantly lower Treg numbers at birth (Table 4).

According to our adjusted logistic regression models, children with lower Treg numbers in cord blood had a significantly higher risk to develop an atopic dermatitis in the first year of life (physician-diagnosed: adjusted OR 1.55, 95% CI 1.00-2.41, $P=0.048$, Figure 4, supplementary Table S5). Upon analysis of specific IgE levels in the sera of the one year old children we found a higher risk for sensitisation against food allergens in the case of lower Treg numbers at birth (adjusted OR 1.55, 95% CI 1.06-2.25, $P=0.020$, Figure 4, supplementary Table S5).

Relationship between maternal history of atopy, environmental exposure and maternal cytokine production during pregnancy

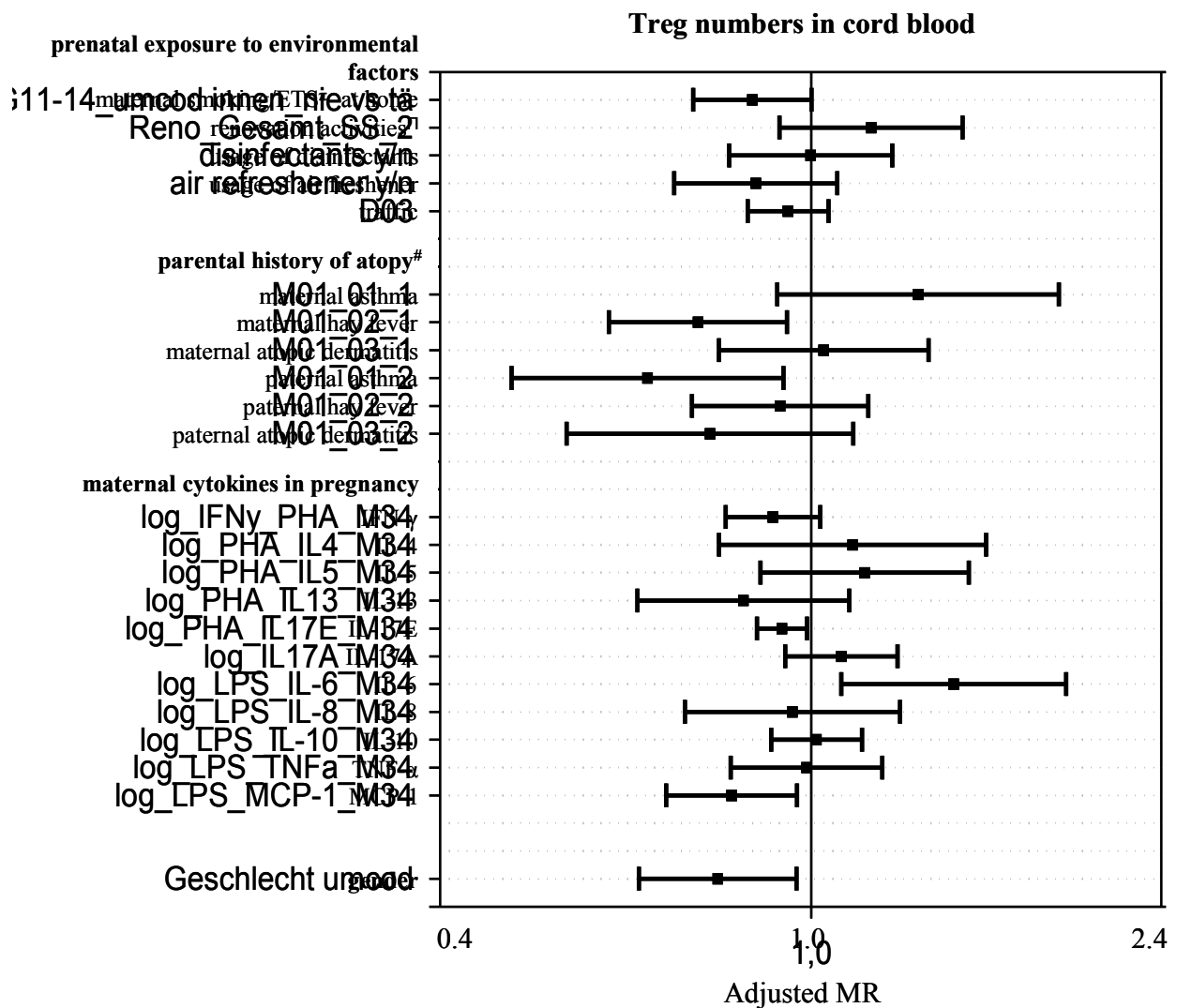
Mothers with an atopic dermatitis ($n=57$) had increased production of IL-5 in PHA stimulated blood samples ($P=0.031$, Mann-Whitney U-Test, supplementary Table S6). Increased IL-13 production was observed in mothers having hay fever ($n=113$, $P=0.044$, Mann-Whitney U-Test, supplementary Table S6) or asthma ($n=34$, $P=0.008$, Mann-Whitney U-Test, supplementary Table S6). Maternal history of atopy (defined as occurrence of asthma or atopic dermatitis or hay fever) was also significantly associated with higher IL-13 production ($n=151$, $P=0.005$, Mann-Whitney U-Test, supplementary Table S6). Environmental exposure was also related to altered cytokine production in maternal blood. Maternal smoking/ETS exposure at home was associated with lower IL-17A production ($n=22$, $P=0.047$, supplementary Table S7). Usage of disinfectants was associated with higher IL-17E production in maternal blood ($n=219$, $P=0.036$, supplementary Table S7). Regarding

inflammatory parameters in stimulated blood samples, only renovation activities during pregnancy were associated with lower IL-8 production ($n=245$, $P=0.003$, data not shown).



Supplementary Fig. S1. Flow chart of the LINA study population with the analysed subcohort.

Relationship between prenatal factors and Treg numbers in cord blood



Supplementary Figure S2: Regression model with all factors possibly influencing cord blood Treg numbers. In addition to the factors shown data were further adjusted for parental educational level and mode of birth. [†]ETS, Environmental tobacco smoke; [‡]refers to questionnaire reported painting, installation of new furniture or changing the flooring during the last twelve month before the birth of the child; [#]History of atopy is defined as: occurrence of asthma or atopic dermatitis or hay fever

Supplementary Table S1. Relationship between parental history of atopy and Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.[#]

Parental history of atopy vs. Treg numbers in cord blood				
	Crude MR	<i>P</i> value	Adjusted MR*	<i>P</i> value [‡]
Parental history of atopy[‡]				
single positive	0.88 (0.73-1.07)	0.201	0.82 (0.68-1.00)	0.048
double positive	0.82 (0.65-1.03)	0.084	0.78 (0.61-0.99)	0.045
Mother				
history of atopy [‡]	0.92 (0.78-1.08)	0.322	0.88 (0.74-1.04)	0.144
asthma	0.98 (0.74-1.30)	0.907	0.98 (0.74-1.30)	0.922
hay fever	0.83 (0.70-1.00)	0.047	0.81 (0.68-0.97)	0.026
atopic dermatitis	1.04 (0.83-1.30)	0.696	1.00 (0.79-1.25)	0.996
Father				
history of atopy [‡]	0.87 (0.73-1.03)	0.102	0.85 (0.71-1.01)	0.071
asthma	0.61 (0.46-0.81)	<0.001	0.60 (0.45-0.81)	<0.001
hay fever	0.89 (0.74-1.07)	0.226	0.90 (0.74-1.09)	0.282
atopic dermatitis	0.80 (0.59-1.08)	0.140	0.74 (0.54-1.01)	0.057

MR, mean ratio

[#]Analysis were performed using a linear regression model with log transformed data

*Adjusted mean ratios for gender, maternal smoking/ETS exposure at home and parental educational level

[‡]Values in boldface are significant ($P < 0.05$)

[‡]History of atopy is defined as: occurrence of asthma or atopic dermatitis or hay fever.

Supplementary Table S2. Relationship between maternal prenatal exposure and Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.[#]

maternal prenatal exposure vs. Treg numbers in cord blood				
	Crude MR	<i>P</i> value	Adjusted MR*	<i>P</i> value ^z
Maternal smoking/ETS exposure at home	0.92 (0.82-1.03)	0.163	0.89 (0.79-1.00)	0.047
Renovation activities [†]	1.05 (0.86-1.29)	0.578	1.06 (0.87-1.29)	0.533
Usage of disinfectants	0.86 (0.73-1.03)	0.109	0.90 (0.76-1.07)	0.257
Usage of air freshener	0.90 (0.76-1.07)	0.237	0.88 (0.74-1.05)	0.167
Traffic	0.98 (0.90-1.06)	0.686	0.97 (0.90-1.06)	0.587

MR, mean ratio

[#]Analysis were performed using a linear regression model with log transformed data

*Adjusted mean ratios for gender, parental history of atopy, parental educational level and maternal production of IL-13 IL-17E, IFN- γ upon PHA stimulation

^zValues in boldface are significant ($P < 0.05$)

[†]refers to the questionnaire reported painting, installation of new furniture or changing the flooring during the last twelve months before the birth of the child

Supplementary Table S3. Relationship between PHA-/LPS-stimulated cytokine production in maternal blood during pregnancy and number of Tregs in cord blood as detected by demethylation of TSDR in the *FOXP3* gene.[#]

cytokine production in maternal blood vs. Treg numbers in cord blood				
Cytokine production in maternal blood samples (34 th gest. week)	Crude MR	<i>P</i> value	Adjusted MR*	<i>P</i> value ^x
Th1/Th2/Th17				
IFN-γ	0.91 (0.85-0.98)	0.024	0.90 (0.83-0.98)	0.020
IL-4	0.88 (0.76-1.02)	0.102	0.90 (0.77-1.05)	0.183
IL-5	1.12 (0.93-1.34)	0.211	1.06 (0.88-1.28)	0.486
IL-13	0.83 (0.74-0.94)	0.004	0.85 (0.75-0.96)	0.009
IL-17E	0.92 (0.87-0.97)	0.002	0.92 (0.87-0.97)	0.003
IL-17A	1.09 (0.97-1.23)	0.132	1.12 (0.99-1.27)	0.053
Inflammatory cytokines				
IL-6	1.14 (0.95-1.37)	0.125	1.13 (0.94-1.36)	0.178
IL-8	0.91 (0.77-1.08)	0.305	0.90 (0.76-1.07)	0.249
IL-10	1.01 (0.93-1.09)	0.774	0.99 (0.91-1.08)	0.912
TNF-α	0.90 (0.78-1.03)	0.143	0.91 (0.79-1.06)	0.238
MCP-1 (CCL2)	0.94 (0.83-1.04)	0.386	0.91 (0.80-1.03)	0.163

MR, mean ratio

[#]Analysis were performed using a linear regression model with log transformed data

*Adjusted mean ratios for gender, parental history of atopy, maternal smoking/ETS exposure at home and parental educational level

^xValues in boldface are significant ($P < 0.05$)

Supplementary Table S4. Cytokine production during pregnancy in PHA-/LPS-stimulated blood samples. Values are presented as medians and the interquartile range.

Maternal cytokine production (34th gest. week)	Concentration in pg/ml Median (IQR)
Th1/Th2/Th17	
IFN-γ	407.60 (233.36-806.36)
IL-4	15.17 (10.80-20.94)
IL-5	3.29 (2.20-4.01)
IL-13	21.46 (14.45-32.37)
IL-17E	105.48 (35.80-265.33)
IL-17A	82.40 (51.98-125.23)
Inflammatory cytokines	
IL-6	9978.35 (7247.99-13142.36)
IL-8	2945.32 (2247.59-4373.74)
IL-10	12.43 (6.33-24.83)
TNF-α	1883.12 (1319.18-2896.18)
MCP-1 (CCL2)	395.95 (268.74-594.26)

Supplementary Table S5. Relationship between Treg numbers in cord blood as detected by demethylation of TSDR in the *FOXP3* gene and the development of allergy as well as sensitisation to food or inhalant specific allergens in the first year of life.[#]

Outcome at age one	Treg numbers in cord blood			
	Crude OR (95% CI)	<i>P</i> value	Adjusted OR (95% CI)	<i>P</i> value
Atopic diseases*				
atopic dermatitis				
symptoms	1.53 (1.03-2.28)	0.034	1.60 (1.04-2.45)	0.028
diagnosed	1.42 (0.94-2.15)	0.087	1.55 (1.00-2.41)	0.048
cradle cap	1.07 (0.80-1.42)	0.616	1.10 (0.81-1.48)	0.518
Sensitisation^{μ, †}				
to food allergens	1.45 (1.01-2.07)	0.040	1.55 (1.06-2.25)	0.020
to inhalant allergens	1.23 (0.63-2.41)	0.526	1.39 (0.69-2.81)	0.351

[#]Analysis was performed using a logistic regression model with log transformed Treg values; values in boldface are significant ($P < 0.05$).

*Adjusted odd ratios for gender, maternal atopic dermatitis, maternal smoking/ETS exposure at home and parental educational level.

^μ Adjusted odd ratios for gender, parental history of atopy and parental educational level. Due to the distribution of cases maternal smoking/ETS exposure could not be considered.

[†] According to the Pharmacia CAP System concentrations >0.35 kU/l were regarded as positive.

Supplementary Table S6a. Association between maternal history of atopy and PHA induced maternal cytokine production in pregnancy. Cytokine concentrations are presented as medians and interquartile range (IQR).[#]

Parameter	Cytokine production in the 34 th gest. week (pg/ml)					
	Maternal history of atopy [‡]		<i>P</i> value	Maternal asthma		<i>P</i> value
	Median (IQR)			Median (IQR)		
	negative (195)	positive (151)	no (311)	yes (34)		
IFN- γ	398.56 (213.46-839.24)	424.03 (257.28-757.01)	0.678	404.57 (220.73-806.36)	459.88 (291.40-824.95)	0.437
IL-4	14.74 (10.45-20.12)	15.74 (11.22-22.70)	0.067	15.00 (10.70-20.45)	17.11 (13.15-24.11)	0.095
IL-5	3.22 (2.14-3.93)	3.49 (2.35-4.08)	0.154	3.25 (2.14-3.97)	3.72 (2.55-4.17)	0.091
IL-13	19.59 (13.24-28.61)	25.24 (15.76-35.36)	0.005	20.48 (14.17-30.84)	30.02 (21.34-38.13)	0.008
IL-17E	90.93 (26.10-229.59)	109.78 (48.57-322.78)	0.249	104.97 (32.19-245.75)	106.57 (48.57-538.27)	0.307
IL-17A	80.05 (47.82-124.70)	83.91 (53.79-131.52)	0.334	83.03 (52.45-125.35)	72.87 (50.98-123.56)	0.645

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

[‡]History of atopy is defined as: occurrence of asthma or atopic dermatitis or hay fever.

Supplementary Table S6b. Association between maternal history of atopy and PHA induced maternal cytokine production in pregnancy. Cytokine concentrations are presented as medians and interquartile range (IQR).[#]

Parameter	Cytokine production in the 34 th gest. week (pg/ml)					
	Maternal hay fever		<i>P</i> value	Maternal atopic dermatitis		<i>P</i> value
	Median (IQR)			Median (IQR)		
	no (231)	yes (113)		no (287)	yes (57)	
IFN- γ	427.55 (213.46-838.06)	401.64 (257.28-750.49)	0.881	407.71 (217.77-812.19)	402.71 (278.83-587.59)	0.995
IL-4	14.86 (10.45-20.45)	15.68 (11.75-22.11)	0.101	15.00 (10.70-20.60)	15.65 (11.17-22.40)	0.661
IL-5	3.25 (2.10-4.06)	3.31 (2.49-3.98)	0.544	3.22 (2.14-3.93)	3.71 (2.88-4.26)	0.031
IL-13	20.41 (13.27-30.62)	24.93 (15.98-33.76)	0.044	20.67 (14.44-30.84)	23.59 (13.93-34.84)	0.260
IL-17E	91.88 (25.51-265.33)	109.78 (52.94-275.31)	0.343	101.85 (38.04-265.33)	109.78 (24.70-261.85)	0.925
IL-17A	78.67 (48.38-125.23)	88.02 (56.98-131.52)	0.226	82.64 (51.98-123.42)	78.61 (52.45-132.20)	0.761

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

Supplementary Table S7a. Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR).[#]

Parameter	Cytokine production in the 34 th gest. week (pg/ml)					
	maternal smoking/ ETS ^π exposure at home		<i>P</i> value	Any renovation [†]		<i>P</i> value
	Median (IQR)			Median (IQR)		
	never (303)	daily (22)	no (76)	yes (245)		
IFN-γ	424.04 (247.46-812.19)	351.14 (170.69-652.26)	0.212	410.81 (244.38-729.62)	435.66 (247.46-838.06)	0.702
IL-4	15.53 (10.79-21.26)	12.60 (8.45-20.12)	0.188	15.69 (10.28-24.12)	15.13 (10.87-20.45)	0.474
IL-5	3.29 (2.19-4.06)	3.18 (1.50-3.89)	0.413	3.36 (2.23-4.12)	3.22 (2.19-3.93)	0.303
IL-13	21.58 (14.94-31.93)	19.58 (11.95-32.42)	0.461	20.26 (15.22-34.73)	21.58 (13.31-31.49)	0.690
IL-17E	101.85 (40.04-275.31)	85.67 (15.86-244.11)	0.674	103.41 (54.92-270.32)	110.20 (40.04-275.33)	0.925
IL-17A	83.91 (54.22-127.05)	55.04 (36.75-86.65)	0.047	78.61 (49.91-130.87)	83.70 (52.47-122.56)	0.959

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

^πETS; environmental tobacco smoke

[†]refers to questionnaire reported painting, installation of new furniture or changing the flooring either before conception or during pregnancy

Supplementary Table S7b. Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR).[#]

Parameter	Cytokine production in the 34 th gest. week (pg/ml)					
	Usage of disinfectants		<i>P</i> value	Usage of air freshener		<i>P</i> value
	Median (IQR)			Median (IQR)		
	no (121)	yes (219)	no (137)	yes (205)		
IFN- γ	380.27 (205.97-841.79)	431.49 (249.84-803.98)	0.474	461.78 (208.28-852.22)	404.57 (249.84-762.82)	0.784
IL-4	14.59 (10.64-19.16)	15.65 (11.22-21.93)	0.128	15.85 (11.53-20.91)	14.80 (10.70-21.26)	0.403
IL-5	3.31 (2.27-4.00)	3.29 (2.19-4.02)	0.937	3.41 (2.19-4.08)	3.29 (2.27-3.93)	0.625
IL-13	19.50 (13.27-30.18)	22.91 (15.51-32.69)	0.050	21.11 (13.93-30.62)	21.66 (14.74-33.21)	0.761
IL-17E	86.89 (25.15-192.85)	112.15 (48.57-310.06)	0.036	125.34 (51.43-297.98)	88.64 (26.10-231.29)	0.233
IL-17A	84.60 (55.18-123.42)	80.25 (50.26-126.62)	0.598	86.38 (55.72-141.79)	75.64 (47.50-120.26)	0.050

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

Supplementary Table S7c. Association between exposures during pregnancy and maternal cytokine production upon PHA-stimulation. Cytokine concentrations are presented as medians and interquartile range (IQR).[#]

concentrations are presented as medians and interquartile range (IQR).			
Parameter	Cytokine production in the 34 th gest. week (pg/ml)		<i>P</i> value
	Traffic*		
	Median (IQR)		
	<10 m (39)	>=150 m (119)	
IFN- γ	354.91 (242.69-864.72)	407.49 (237.99-872.49)	0.715
IL-4	13.33 (11.52-22.11)	15.67 (11.66-21.86)	0.434
IL-5	3.16 (1.50-3.84)	3.56 (2.35-4.12)	0.179
IL-13	21.97 (15.78-30.34)	22.65 (13.92-34.27)	0.638
IL-17E	126.44 (24.77-303.92)	91.88 (43.86-209.16)	0.412
IL-17A	73.32 (48.38-125.23)	91.26 (53.91-139.83)	0.209

[#]Analyses were performed using Mann-Whitney U-Test. Significant values are in boldface ($P < 0.05$).

*Values are presented in meter and refer to the distance between dwelling and main street.