

TLR2 polymorphisms influence neonatal regulatory T cells depending on maternal atopy

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Abstract

Background: Toll-like receptor (TLR) polymorphisms have been associated with atopic diseases in children and adults. Development of atopic diseases may be modified by TLR-mediated signals that modulate T-regulatory cells (Tregs) early in life when maternal influences are still present and relevant. The aim of this study was to assess whether genetic TLR variants influence Tregs in neonates.

Methods: Twelve single nucleotide polymorphisms located in TLR1, TLR2, TLR4, TLR6, and TLR10 were genotyped in 200 cord blood samples (72 samples from atopic, 128 from nonatopic mothers). Cord blood mononuclear cells were cultured without or with stimulation [lipid A (LpA), peptidoglycan (Ppg), phytohemagglutinin, house dust mite]. mRNA expression of Treg marker genes [forkhead box protein P3 (FOXP3), glucocorticoid-induced tumor necrosis factor receptor (GITR), lymphocyte activation gene 3 (LAG3)], TLR2, Th1/Th2 cytokines, and tumor necrosis factor alpha (TNF- α) was measured.

Results: In children with the AA genotype of the TLR2 promoter variant rs4696480, gene expression of FOXP3 and Treg marker genes GITR and LAG3 as well as Th2 cytokines and TNF- α secretion was significantly increased in the presence of maternal atopy and Tregs decreased without maternal atopy. In carriers of the GG genotype for TLR2 rs1898830, gene expression of Treg marker genes was significantly decreased with and increased without maternal atopy. FOXP3 expression was also modified by TLR1 rs4833095 ($P \leq 0.03$) and trend-wise by TLR10 rs4129009 after LpA and Ppg stimulation.

Conclusions: Genetic variations of TLR2, TLR1, and TLR10 affect Treg marker gene expression in cord blood. Gene-immunological interactions of the TLR pathway influence Tregs early in life, modulated by maternal atopy. This may be relevant for immune maturation in the development of atopic diseases in childhood.

Abbreviations

APC, antigen-presenting cell; CBMCs, cord blood mononuclear cells; CT, cycle threshold; Derp1, dermatophagoides pteronyssinus; FOXP3, forkhead box protein P3; GITR, glucocorticoid-induced tumor necrosis factor receptor; HWE, Hardy-Weinberg Equilibrium; IFN- γ , interferon gamma; IQR, interquartile range; LAG3, lymphocyte activation gene 3; LpA, lipid A; MAF, minor allele frequency; PAMPs, pathogen-associated molecular patterns; PHA, phytohemagglutinin; Ppg, peptidoglycan; SNP, single nucleotide polymorphism; TLR, toll-like receptor; TNF- α , tumor necrosis factor alpha; Tregs, regulatory T cells; WT, wildtype.

Genetic and environmental factors contribute to the development of atopic diseases, most likely as interacting factors. Several studies revealed the impact of genetic polymorphisms on atopic diseases (1–3) and the relevance of gene–environment interactions (4–6). The prevalence of atopic diseases in childhood increased dramatically in recent decades, while westernized lifestyle with improved hygiene standards and reduced microbial exposure evolved (7). The ‘hygiene hypothesis’ proposes microbiological exposure during early life as protective against the development of atopic diseases (8).

The effect is potentially mediated by toll-like receptors (TLRs), which represent an evolutionarily conserved family of innate receptors and recognize pathogen-associated molecular patterns of microbes capable of activating innate and adaptive immunity (9). Toll-like receptors are proposed to directly and indirectly modulate T-regulatory cells (Tregs), an important cell population in humans required for keeping healthy immune responses in balance. Toll-like receptor stimulation is required to induce the expression of costimulatory molecules on antigen-presenting cells and cytokine secretion that tightly control Treg-mediated suppression (10). Additionally, recent publications suggest the presence of an unrecognized pathway by which TLRs expressed on Tregs directly modulate immune responses by causing modulated suppressive activity of Tregs (11–13). Several studies including ours have shown that Tregs play an important role in the development of atopic diseases and that TLR pathways influence the amount and function of Tregs in cord blood (14–18). Thus, TLRs play a key role in recognition of the microbial environment and modulation of allergy-related effects.

Polymorphisms of these receptors may influence the development of atopic diseases as previously shown (1, 5, 19–22). The TLR4 single nucleotide polymorphism (SNP) A896G was associated with hyporesponsiveness to inhaled endotoxins (4), while genetic variation in TLR2 influences the susceptibility to atopic diseases in farmer's children (5). Overall, genetic studies on TLR2 polymorphisms and atopic diseases showed contradictory results (5, 23). Genetic variants of TLR1, TLR6, and TLR10 were associated with asthma development in childhood (24), while the TLR9 polymorphism C-1237T was proposed to affect the susceptibility to atopic eczema (25).

However, immunological mechanisms by which polymorphisms affect atopic disease development are still not well understood. Based on our previous studies, we hypothesize that these mechanisms start early in life (16, 17, 24, 26). Therefore, we studied the immunological effects of TLRs, identified by previous studies to influence asthma and allergy development, in cord blood and investigated their effect on Treg- and Th-cell responses in neonates.

Methods

Human study population

Fetal cord blood ($n = 200$) was obtained from neonates born in the Munich metropolitan area, Germany. Subjects were recruited from July 2005 to September 2007 during the last trimester of pregnancy (details, Data S1). Previous studies showed that maternal atopy is a confounder for Treg-cell number and function (16, 17). Maternal atopy was defined as history of doctor's diagnosis of asthma and/or eczema and/or hay fever ever. During pregnancy, 16% of the mothers suffered from current atopic disease (doctor's diagnosis of current asthma and/or eczema and/or hay fever). Total and specific IgE [radioallergosorbent test (RAST)] was measured. A positive specific IgE was defined as 1 or more positive reactions ≥ 0.35 IU/ml to a panel of 20 common allergens,

and 85% of atopic mothers had a positive RAST test. Non-atopic mothers had no atopic disease and sensitization levels comparable to previous epidemiological studies among non-diseased subjects (27). Potential covariates (Data S1) were reported when they influenced the outcome. The laboratory investigators were blinded to clinical information; samples were analyzed based on sample availability. Informed consent was obtained from the mothers. Approval was obtained from the human ethics committee of the Bavarian Ethical Board, LMU Munich, Germany.

Follow-up at 3 years of age

Follow-up (3 years) is reported in the Data S1.

Polymorphisms selection and genotyping

Data on SNPs for TLR genes were screened for mutations by the Innate Immunity Programs for Genomic Applications (www.innate-immunity.net). Using HAPLOVIEW software (Version 3.32, MIT, www.broad.mit.edu/mpg/haploview), haplotype tagging SNPs were selected for genotyping from all TLR polymorphisms with a minor allele frequency (MAF) > 0.10 . Based on previous experiments, 12 polymorphisms of the human TLR genes TLR1, TLR2, TLR4, TLR6, and TLR10 were selected because of their association with asthma and their putative functional implications (Primers available on request) (5, 24). Genotyping was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom Inc., San Diego, CA, USA) (28). Polymerase chain reaction assays and associated extension reactions were designed with the SPECTRODESIGNER software (Sequenom Inc.). All amplification and extension reaction conditions have been previously described (29). Deviations from Hardy–Weinberg equilibrium (HWE) were assessed for quality control of genotyping procedures.

Isolation, culture, lymphocyte proliferation, and real-time PCR of CBMCs

Cord blood was collected from the umbilical vein after delivery and processed within 24 h. Cord blood mononuclear cells (CBMCs) were isolated by density-gradient centrifugation with Ficoll-Hypaque, cultured without stimulation or stimulated with lipid A (LpA, 0.1 $\mu\text{g/ml}$), peptidoglycan (Ppg, 10 $\mu\text{g/ml}$), phytohemagglutinin (PHA, 5 $\mu\text{g/ml}$), or Dermatophagoides pteronyssinus (Derp1, 30 $\mu\text{g/ml}$) as previously described (17). After incubation for 3 days, gene expression and cytokines were assessed. Details on real-time quantitative RT-PCR is given in Methods Supplement (Data S1).

Cytokine measurements

Supernatants were collected and cytokine concentrations measured with Human Cytokine Multiplex-Assay-kit according to the manufacturer's instructions (Bio-Rad, Munich, Germany). The lowest limits of detection (pg/ml) were 3.0 [tumor necrosis factor alpha (TNF- α)], 2.1 (IL-13), 1.8 (IL-5),

1.3 [interferon gamma (IFN- γ)], and 0.5 [interleukin-12 (IL-12)]. Nondetectable concentrations were assigned to a value of 0.01 for inclusion into the analysis.

Statistical analysis

Data analysis was performed with sas 9.2 (SAS Institute, Cary, NC, USA). Data for frequency of SNPs and genetic analyses were analyzed with chi-square test (two groups) or Mantel-Haenszel test for trend (three groups). A recessive model was used to compare the outcome in carriers of the SNP with carriers of the heterozygous genotype and the wildtype.

Data of cytokine concentrations and gene expression, which were not normally distributed, were analyzed with Mann-Whitney rank test (two groups) or Kruskal-Wallis rank test (three groups) and were reported as median (25–75% interquartile range). Rank-transformed data of gene expression were additionally analyzed by linear regression models, treating allele count as a linear variable. Statistical significance was defined as $P < 0.05$.

Results

Genetic variants of TLR 2 (rs4696480, rs1898830) modify mRNA expression of Treg marker genes in CBMCs according to maternal atopy

Twelve SNPs were selected for genotyping based on their involvement in allergy development (summary Table 1) (5, 24). Success rate for genotyping was $>97\%$. None of the SNPs deviated from HWE.

Single nucleotide polymorphism effects on Treg marker gene expression [forkhead box protein P3 (FOXP3), gluco-

corticoid-induced tumor necrosis factor receptor (GITR), lymphocyte activation gene 3 (LAG3)] were determined, whereas FOXP3 represents the most specific marker for natural Tregs. Forkhead box protein P3 mRNA expression after LpA and Ppg stimulation was significantly lower for TLR1 SNP rs4833095 and lower for TLR10 SNP rs4129009 (overview Table 2; data Table S2). Forkhead box protein P3 mRNA expression after PHA stimulation was decreased in TLR2 SNP rs3804099 and in TLR4 SNP rs10759932. There were no additional significant findings for TLR4 or TLR6 SNPs. In Figs 1, 2, S1 and S2, the y-axis was depicted inversely as mRNA expression was described as Δ ct; higher Δ ct values correspond to lower gene expression.

Genetic variants of TLR2 rs4696480 and rs1898830 increased or decreased the expression of FOXP3 and two other Treg marker genes (GITR, LAG3) in the opposite way, depending on maternal atopy. As maternal atopy was identified as important confounder and differences in allele frequency were detected for both TLR2 SNPs (rs4696480, rs1898830) depending on maternal atopy (Table S3), expression of Treg markers was assessed for CBMCs of mothers with and without atopy (Table 2, Figs 1 and 2) (16, 17). There was no significant influence of rs4696480 and rs1898830 SNPs on the expression of Treg markers, if all cord blood samples were analyzed together. The other investigated TLR SNPs were not significantly influenced by maternal atopy.

When stratified for maternal atopy, the SNPs rs4696480 (T/A, MAF 0.54) and rs1898830 (A/G, MAF 0.33) showed differences in allele frequencies. The frequency of AA genotypes was significantly higher relatively to AT/TT and AG/GG, respectively, in neonates with compared to no maternal atopy (Table S3, $P \leq 0.04$). These effects were not mediated by differences in MAFs in atopic compared with nonatopic

Table 1 Biological characteristics of the analyzed TLR SNPs

Gene	rs no.	Position relative to ATG*	Alleles	Location	Δ AA	Δ TF	ESE/ESS	MAF [ISAAC Munich†]	MAF [our cohort]
TLR1	rs4833095	743	A/G	CDS	X		X	0.26	0.29
TLR1	rs5743595	-2192	T/C	Intron		X		0.20	0.21
TLR2	rs1898830	-15607	A/G	Promoter		X		0.33	0.33
TLR2	rs3804100	1350	T/C	CDS			X	0.08	0.07
TLR2	rs3804099	597	T/C	CDS			X	0.45	0.43
TLR2	rs4696480	-16934	T/A	Promoter				0.51	0.54
TLR4	rs4986790	4435	A/G	CDS	X	X	X	0.04‡	0.04
TLR4	rs4986791	4735	C/T	CDS	X		X	0.06	0.06
TLR4	rs6478317	-6687	A/G	Promoter		X		0.29	0.32
TLR4	rs10759932	-5724	T/C	Promoter		X		0.12	0.14
TLR6	rs5743789	-2079	T/A	Promoter				0.22	0.22
TLR10	rs4129009	2323	A/G	CDS	X			0.19	0.21

CDS, Coding sequence; MAF, minor allele frequency; Δ AA, SNP leads to an amino acid change; Δ TF, SNP changes transcription factor binding based on FastSNP analysis; ESE/ESS, SNP changes exonic splicing enhancer or exonic splicing silencer based on FastSNP analysis; TLR, toll-like receptor.

*Based on March 2006 human reference (NCBI build 36.1); SNP, single nucleotide polymorphism.

†ISAAC Munich, Munich study population of the International study of asthma and allergies in childhood, case-control study.

‡Not genotyped in Munich, HapMap data.

Table 2 Influence of the 12 assessed SNPs on gene expression of Treg-associated genes and Th1, Th2, and TNF- α cytokine secretion

Gene	rs number SNP		Tregs			Th1	Th2	TNF- α
			FOXP3	GITR	LAG3			
TLR1	rs4833095 GG	all	↓ LpA* Ppg*	n.d.	n.d.	n.d.	n.d.	↑ Derp1*
TLR1	rs5743595 CC	all	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TLR2	rs1898830 GG	CBMCs [A]	↓ U* Derp1*	↓ U* Derp1	↓ U* PHA* Ppg Derp1	n.d.	↑ IL-13: U* PHA LpA	↑ PHA LpA* Derp1*
		CBMCs [NA]	↑ U* LpA*	↑ U* LpA* Ppg* Derp1	↑ U* LpA* Ppg*	n.d.	n.d.	n.d.
TLR2	rs4696480 AA	CBMCs [A]	↑ U* Derp1*	↑ U Ppg* Derp1*	↑ U* PHA Derp1	n.d.	↑ IL-5: PHA* LpA IL-13: PHA* LpA* Derp1*	↑ PHA LpA Derp1
		CBMCs [NA]	↓ U* PHA* LpA* Ppg	↓ U* PHA Ppg* Derp1*	↓ U* PHA LpA Ppg* Derp1	n.d.	n.d.	n.d.
TLR2	rs3804100 CC	all	n.d.	n.d.	↑ Derp1*	n.d.	n.d.	↑ U*
TLR2	rs3804099 CC	all	↓ PHA*	n.d.	n.d.	n.d.	n.d.	↑ U*
TLR4	rs4986790 GG	all	n.d.	n.d.	n.d.	n.d.	↑ IL-13: Derp1*	n.d.
TLR4	rs4986791 TT	all	n.d.	n.d.	n.d.	n.d.	↑ IL-13: Derp1*	n.d.
TLR4	rs6478317 GG	all	n.d.	n.d.	n.d.	↑ IL-12: LpA*	n.d.	n.d.
TLR4	rs10759932 CC	all	↓ PHA*	n.d.	n.d.	n.d.	n.d.	n.d.
TLR6	rs5743789 AA	all	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TLR10	rs4129009 GG	all	↓ LpA Ppg	n.d.	n.d.	n.d.	n.d.	n.d.

↑, expression upregulated; ↓, expression downregulated; *Th1*, secretion of IFN- γ and IL-12; *Th2*, secretion of IL-5 and IL-13; *TNF- α* , secretion of TNF- α ; *U*, unstimulated; *PHA*, phytohemagglutinin; *LpA*, lipid A; *Ppg*, peptidoglycan; *Derp1*, dermatophagoides pteronyssinus; *CBMCs*, cord blood mononuclear cells; *CBMCs [A]*, CBMCs of atopic mothers; *CBMCs [NA]*, CBMCs of mothers without atopy; *all*, no differentiation according to maternal atopy; *n.d.*, no difference; FOXP3, forkhead box protein P3; GITR, glucocorticoid-induced tumor necrosis factor receptor; IFN- γ , interferon gamma; LAG3, lymphocyte activation gene 3; SNP, single nucleotide polymorphism; TLR, toll-like receptor; TNF- α , tumor necrosis factor alpha.

* $P < 0.05$.

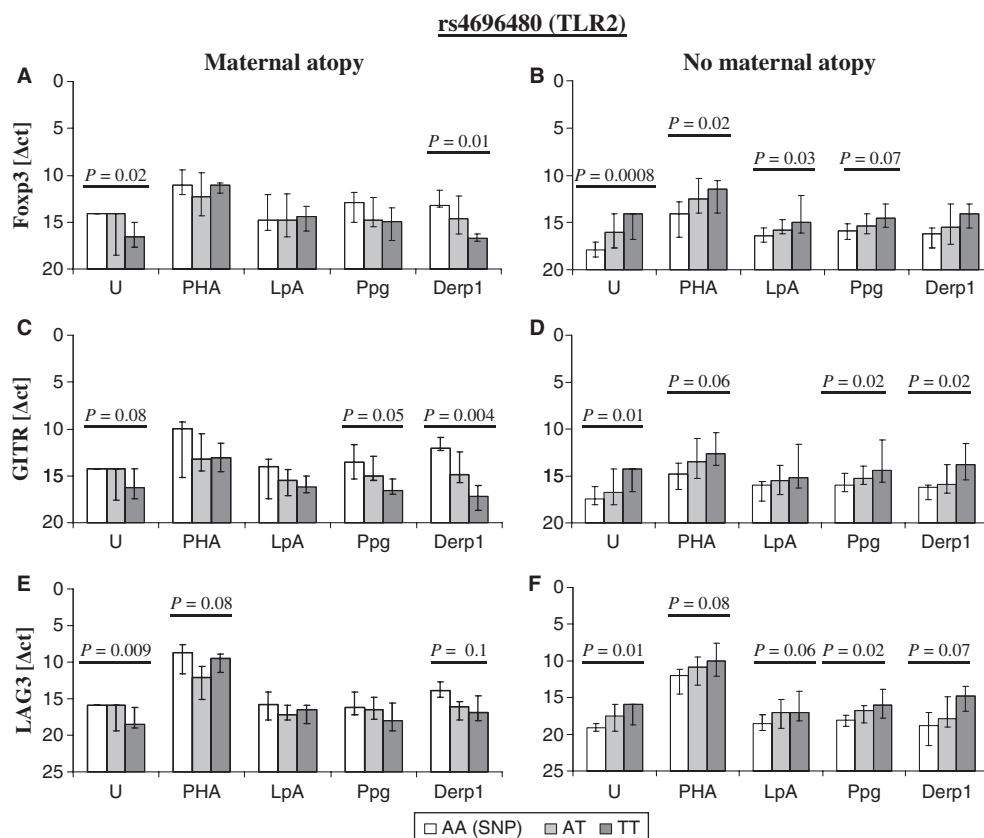


Figure 1 Association of rs4696480 with forkhead box protein P3, glucocorticoid-induced tumor necrosis factor receptor, lymphocyte activation gene 3 gene expression, unstimulated (U), following stimulation (phytohemagglutinin, lipid A, peptidoglycan, dermatophagoides pteronyssinus). Values were presented as Δ ct. Higher values reflect lower expression (axis *vice versa*). Kruskal–Wallis test

was performed to compare AA (single nucleotide polymorphism), AT, and TT. Median (interquartile range); *n* (maternal atopy) = 15(AA), 16(AT), 8(TT); *n* (no maternal atopy) = 16(AA), 33(AT), 18(TT). Gene expression in CBMC of atopic (A, C, E) and nonatopic mothers (B, D, F) for FOXP3, GITR, LAG3.

mothers or fathers (not shown), indicating no genetic influence. In CBMCs of atopic mothers, the AA genotype (rs4696480) showed higher FOXP3, GITR, and LAG3 expression in unstimulated cells (Fig. 1A,C,E, $P = 0.02$, 0.08 , 0.009) and after Derp1 stimulation ($P = 0.01$, 0.004 , 0.1) and nonsignificantly higher expression after PHA, LpA and Ppg stimulation, compared with AT and TT; data were similar applying a recessive model (Data S1). In offspring of nonatopic mothers, CBMC gene expression of FOXP3, GITR, and LAG3 was significantly or borderline lower with AA genotype after all stimulation conditions (Fig. 1B,D,F) compared with AT and TT.

The TLR2 rs1898830 GG genotype showed lower FOXP3, GITR, and LAG3 expression in unstimulated CBMCs from neonates with atopic mothers (Fig. 2A,C,E, $P \leq 0.04$) and after Derp1 stimulation (Fig. 2A,C,E, $P = 0.05$, 0.08 , 0.06), compared with AG and AA; data were similar applying a recessive model (Data S1). Opposite effects were observed in unstimulated CBMCs from nonatopic mothers (Fig. 2B,D,F, all $P \leq 0.02$) or after LpA, Ppg ($P \leq 0.04$; except for FOXP3_Ppg), or Derp1 stimulation for GITR ($P = 0.09$).

Impact of genetic TLR variants on Th-cell cytokine secretion

Effects were not restricted to Treg marker expression. Th2 cytokine and TNF- α secretion were also altered by the same TLR2 rs4696480 and rs1898830 alleles. In CBMCs of atopic mothers with TLR2 rs4696480 AA, after PHA, LpA, and Derp1 stimulation, significantly or borderline higher IL-5 (Fig. 3A, PHA $P = 0.04$, LpA $P = 0.06$), IL-13 (Fig. 3C, $P = 0.005$, 0.02 , 0.04), and TNF- α (Fig. 3G, $P = 0.07$, 0.07 , 0.06) secretion was detected. For IFN- γ , no differences were observed. Tumor necrosis factor alpha concentrations of AA carriers of rs4696480 were higher in the CBMCs of atopic vs nonatopic mothers after PHA, LpA, and Derp1 stimulation (not shown, $P = 0.006$, 0.05 , 0.007). For rs4696480, no differences in IL-5, IL-13, IFN- γ , and TNF- α secretion were detected if mothers were nonatopic (not shown).

In CBMCs of atopic mothers with rs1898830 (TLR2), no differences in IL-5 and IFN- γ secretion were seen depending on the genotype. IL-13 secretion was lower in rs1898830 GG before and after PHA and LpA stimulation (Fig. 3D, $P = 0.02$, 0.08 , 0.08). For TNF- α , there were significant

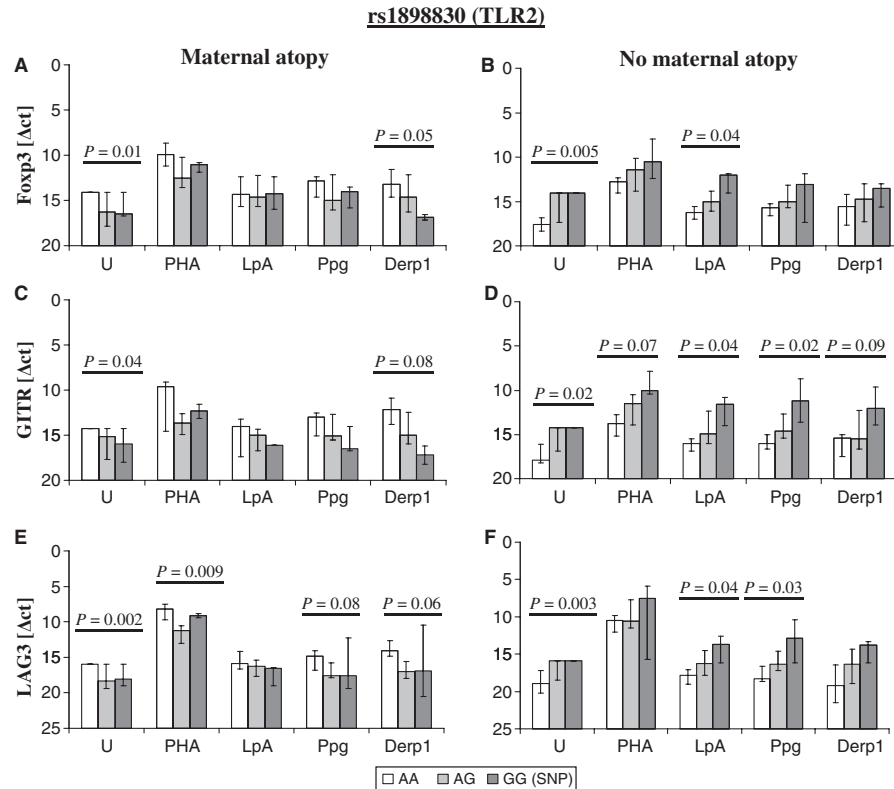


Figure 2 The influence of the rs1898830 genotype on forkhead box protein P3, glucocorticoid-induced tumor necrosis actor receptor, and lymphocyte activation gene 3 gene expression, unstimulated (U), and following stimulation (phytohemagglutinin, lipid A, peptidoglycan, dermatophagoides pteronyssinus). Values were presented as Δ Act. Kruskal–Wallis test was used to compare GG

(single nucleotide polymorphism), AG, and AA. Data were shown as median (interquartile range); n (maternal atopy) = 15(AA), 11(AG), 3(GG); n (no maternal atopy) = 11(AA), 28(AG), 5(GG). Gene expression in CBMC of atopic (A, C, E) and nonatopic mothers (B, D, F) for FOXP3, GITR, LAG3.

effects for LpA-, Ppg-, and Derp1-stimulated cells (Fig. 3H, $P = 0.03, 0.04, 0.01$). In CBMCs with rs1898830 (TLR2), no differences in IL-5, IL-13, IFN- γ , and TNF- α -secretion were detected depending on the genotype in nonatopic mothers (not shown). Cytokine secretion depending on TLR4 SNPs is listed in the Data S1.

The influence of genetic variations of rs4696480 and rs1898830 on TLR2 gene expression

We hypothesized that the effects of TLR2 SNPs may be attributed to allele-specific changes in TLR2 expression. In the CBMCs of carriers of TLR2 rs4696480 AA, TLR2 expression (LpA) was lower compared with AT and TT, if mothers were atopic (Fig. 4A). Cord blood mononuclear cells of nonatopic mothers (rs4696480 AA) showed significantly increased TLR2 expression before and after PHA stimulation (Fig. 4B, both $P \leq 0.05$). If TLR2 expression was analyzed in all samples independent of maternal atopy, no differences in gene expression were detected comparing AA, AT, and TT (Fig. 4E). For CBMCs with TLR2 rs1898830 GG, TLR2 expression was not significantly different compared with AG and AA (Fig. 4B,D,F).

Impact of genetic TLR variants on clinical outcomes

The TLR2 SNP rs1898830 showed protection against wheeze, yet not significant (Table S4; $P = 0.07$). If both TLR2 SNPs rs1898830 and rs4696480 were stratified for maternal atopy, carriers of the TLR2 SNP rs1898830 without maternal atopy showed protection against wheeze (Table S5; $P = 0.07$) and against any atopy ($P = 0.11$); however, data were not significant.

Carriers of the rs4696480 SNP with maternal atopy showed protection against any atopy (Table S5; $P = 0.11$), while subjects without maternal atopy had a risk for food allergy ($P = 0.10$); yet data did not reach significance.

The TLR4 SNP rs6478317 presented as a significant risk factor for food allergy (Table S4; $P = 0.0006$). Other TLR SNPs showed an increased risk for atopic dermatitis (TLR4, rs4986790, rs4986791; TLR6, rs5743789; $P \leq 0.09$) or an increased risk for any atopy (TLR1, rs5743595; $P = 0.1$) (Data S1).

Discussion

We assessed the influence of 12 polymorphisms (SNPs) within TLR genes on Tregs in CBMCs. Effects on Treg marker gene

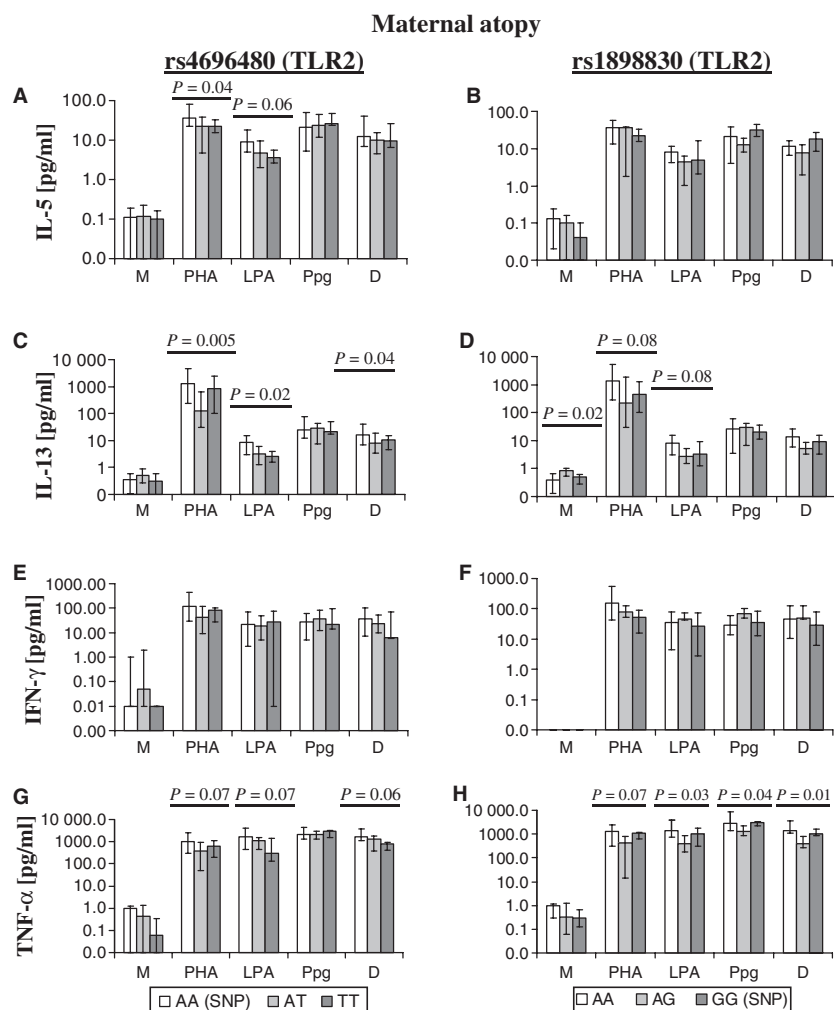


Figure 3 The influence of the rs4696480 and rs1898830 genotypes on concentrations of IL-5, IL-13, interferon gamma (IFN- γ), and tumor necrosis factor alpha in the cord blood of atopic mothers (pg/ml). Kruskal–Wallis test was used to compare single nucleotide polymorphism, heterozygous genotype, and wildtype. Data were

shown as median (interquartile range). Y-axis was depicted in logarithmic scale to show ranges after different stimulations. rs4696480: $n = 27$ (AA), 30 (AT), 10 (TT). rs1898830: $n = 24$ (AA), 12 (AG), 4 (GG). (A–H) Cytokine secretion in CBMC of atopic mothers in pg/ml.

expression (FOXP3, GITR, LAG3) were primarily observed for TLR2 SNPs (rs4696480, rs1898830).

Depending on the atopy status of the mother, Treg marker genes were increased or decreased in the opposite way for both TLR2 genotypes. Th2 secretion and TNF- α were influenced by TLR2, TLR1, and TLR4 SNPs. For TLR1 (rs4833095) and TLR10 (rs4129009) SNPs, FOXP3 mRNA expression was decreased upon innate stimulation.

Several studies proposed a direct or indirect modulation of Tregs by TLRs via antigen presentation and subsequent downstream activation of cytokine secretion, regulation of Treg suppression, or direct modulation of TLRs on Tregs (10–13). The diametrical effect on Treg markers among genetic variants of rs4696480 and rs1898830 (TLR2) in the CBMCs of atopic compared with nonatopic mothers in our study is important.

This may point to an immunomodulatory effect through maternal atopy, potentially setting the immunological basis

at birth, by either direct intrauterine immunological pathways, epigenetic, or currently unknown mechanisms, including genetic and immune-mediated modulation.

In our study, maternal atopy reflects a heterogeneous phenotype including maternal asthma, hay fever, and/or eczema. One could presume that the observed differences according to maternal atopy may be caused by the fact that in atopic mothers an excess of TLR risk alleles associated with atopy may be found. Accordingly, the observed differences could be attributed to maternal genotype rather than genotype of the offspring. However, an influence of maternal genotypes on Treg modulation of the fetus is unlikely as no accumulation of risk genotypes was observed in atopic mothers. Influences through epigenetic regulation may partially contribute to our findings but have not been focus of this study and are currently not well understood. Furthermore, epigenetic effects do probably not completely account for the

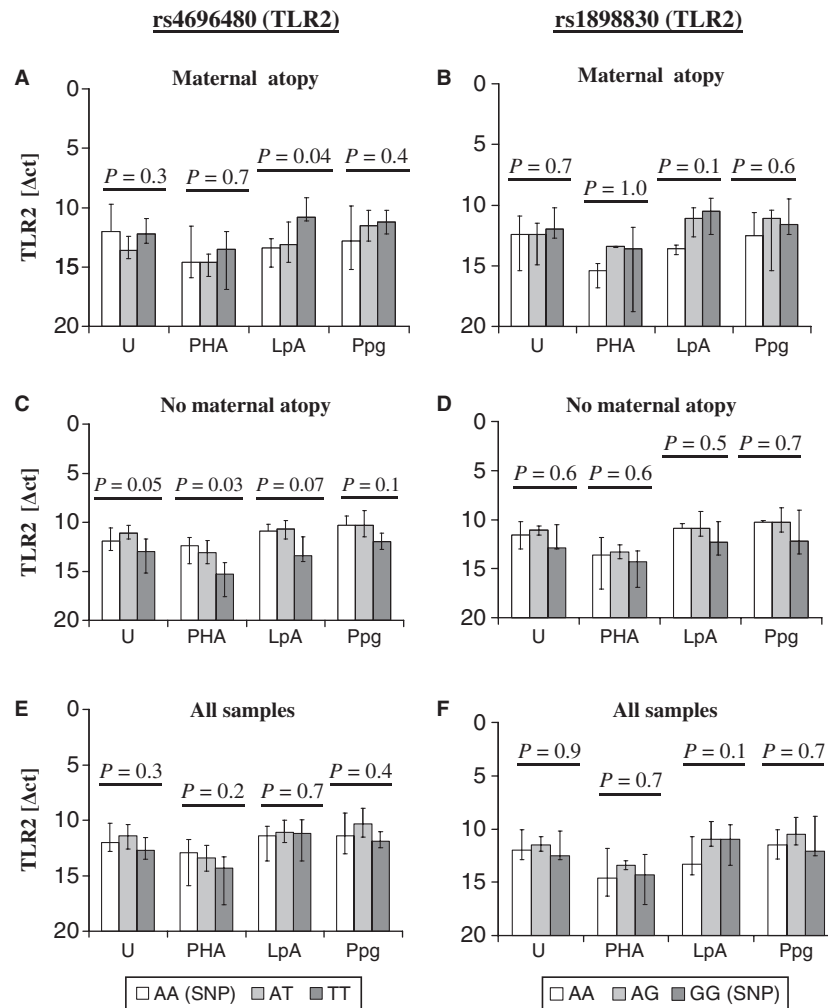


Figure 4 Influence of rs4696480 and rs1898830 on toll-like receptor2 mRNA expression, Δct , median (interquartile range). Kruskal–Wallis test to compare single nucleotide polymorphism with heterozygous/wildtype genotype. rs4696480: (A) *n* (maternal

atopy) = 10(AA), 7(AT), 8(TT). (C) *n* (no maternal atopy) = 9(AA), 17(AT), 8(TT). (E) all. rs1898830: (B) *n* (maternal atopy) = 10(AA), 5(AG), 4(GG). (D) *n* (no maternal atopy) = 8(AA), 7(AG), 3(GG). (F) all.

opposite findings of Tregs through maternal atopy, which is known as a strong risk factor for childhood asthma (30).

In support of our data, Eder et al. (5) described A as risk allele of childhood asthma for the rs4696480 genotypes, however, in farm children. In our study, in CBMCs with AA genotype without maternal atopy, decreased Tregs and no differences in Th2/Th1 cytokines were detected. However, with maternal atopy, increased IL-5, IL-13 (Th2), and pro-inflammatory TNF- α were detected. Several studies associated a Th1/Th2 imbalance with development of atopic manifestation in childhood (31, 32). In parallel with Th2 predominance, increased Treg markers were found in CBMCs with AA genotype and maternal atopy. Conceivably, Tregs may get upregulated early in life to potentially counterbalance Th-cell regulation toward healthy immune development.

One important point needs to be considered as data were not reported after adjustment for multiple testing. Yet if Bonferroni correction was made for two SNP groups and

two clusters of T-cell regulation, data with $P \leq 0.01$ will still be biologically relevant (details in Data S1).

Kormann et al. (24) found the TLR2 SNP rs1898830 to be associated with a trend for nonatopic asthma. In our study, the GG genotype in combination with maternal atopy led to decreased Treg markers, indirectly pointing to a reduced amount of Tregs and potentially insufficient immunosuppression. Kormann et al. (24) showed an effect of TLR2 SNPs with childhood asthma, including rs1898830, as heterodimers with TLR1, TLR6, and TLR10.

Effects of the combination of SNPs on T-cell responses could not be assessed in this study owing to small sample size and limitations in power. However, differences in comparison with Kormann's (24) data may also be explained by time of immune maturation and different study designs. Moreover, changes in Tregs by TLR1, TLR6, and TLR10 might occur in childhood and not early in life.

No effects of other single TLR SNPs on T-cell regulation were identified except for TLR1 (rs4833095), TLR10 (rs4129009), and TLR4 (rs4986790, rs4986791, rs6478317, rs10759932). Parallel to decreased FOXP3 for rs4129009 SNP in our study, a significant association with physician-diagnosed asthma was shown by Lazarus et al. (21). Reduced FOXP3 expression early in life may potentially lead to an insufficient immunosuppression via Tregs later in childhood. Significant effects of TLR1 and TLR10 SNPs were seen following nonspecific LpA and Ppg stimulation for these receptors. As heterodimerization of TLR1 with TLR2 was shown (33) and TLR10, highly homologous to TLR1 and TLR6, was shown to form heterodimers with TLR2 (34), the ligand spectrum might be more complex and nonspecific stimulation through LpA and Ppg may be possible. Analysis of the follow-up at 3 years did not show significant associations between TLR1 and TLR10 genotypes with allergic outcomes. However, the number of cases was limited.

Eder et al. (5) showed that carriers of the T allele of TLR2 rs4696480 were significantly less likely to have atopic diseases, hypothesizing an augmented TLR2 expression in a TLR2-rich microbial environment. Indirectly, the increase in TLR2 in the TT genotype of rs4696480 in CBMCs with maternal atopy would go along with this hypothesis. However, this was not shown for all stimulation conditions, and without maternal atopy, we detected an opposite effect. Compared with Eder's study in farmers' children, we investigated a primarily city-based population in the Munich area (5). Although TLR2 ligands are proposed to directly modulate Treg function by either inhibiting or supporting Treg suppressive activity (35), no clear effect of the genotype on Tregs via TLR2 expression could be demonstrated in our study. Changes in TLR2 expression in relation to both TLR2 SNPs cannot be related to transcription factor binding changes as reported for TLR2 SNP rs1898830 (24) and require further investigation.

Follow-up of all children at 3 years did not show an association of the rs4696480 genotype with the assessed outcomes, while carriers of rs1898830 SNP showed less wheeze.

If both TLR2 SNPs (rs4696480 and rs1898830) were stratified for maternal atopy, clinical outcomes may be explained by cord blood immune responses. Higher Tregs generally corresponded to protection against e.g. any atopy (rs4696480 and rs1898830 SNPs) and against wheeze (rs1898830 SNP) and lower Tregs with risk e.g. for food allergy (rs4696480 SNP).

Whether the TLR genotype of the fetus and maternal atopy allow further prediction for immune maturation regarding atopy development or *vice versa* protection against childhood asthma requires further follow-up of this study later in childhood and of other cohort studies, preferably in a longitudinal manner, as only this can predict later effects during immune maturation, extending our studies on cord blood T-cell responses.

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Author contributions

J. Liu performed experiments, analyzed data, and contributed to the writing of the manuscript. D. Raedler analyzed data and contributed to the writing of the manuscript. S. Illi performed data analysis. Elisabeth Klucker and Elif Turan performed the follow-up assessment. E. v. Mutius and M. Kabesch contributed to data interpretation and writing of the manuscript. B. Schaub has designed the study, performed data analysis and interpretation, and wrote the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>

Data S1. Additional information on methods, results, statistical analysis and discussion.

Figure S1. Association of rs4696480 genotype with forkhead box protein P3, glucocorticoid-induced tumor necrosis factor receptor, lymphocyte activation gene 3 gene expression (recessive model).

Figure S2. Association of rs1898830 genotype with forkhead box protein P3, glucocorticoid-induced tumor necrosis factor receptor, lymphocyte activation gene 3 gene expression (recessive model).

Table S1. mRNA-specific primer pairs for RT-PCR of 18S, forkhead box protein P3, glucocorticoid-induced tumor necrosis factor receptor, lymphocyte activation gene 3, toll-like receptor2.

Table S2. Forkhead box protein P3 mRNA expression of rs4833095 (toll-like receptor1) and rs4129009 (toll-like receptor10) after lipid A and peptidoglycan stimulation.

Table S3. Distribution of genotype frequencies of rs4696480 and rs1898830 (toll-like receptor2) in cord blood mononuclear cells with and without maternal atopy.

Table S4. Effect of toll-like receptor single nucleotide polymorphisms on different clinical outcomes.

Table S5. Effect of the toll-like receptor2 single nucleotide polymorphisms rs4696480 and rs1898830 on different clinical outcomes stratified for maternal atopy.

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Figure S1

rs4696480 (TLR2)

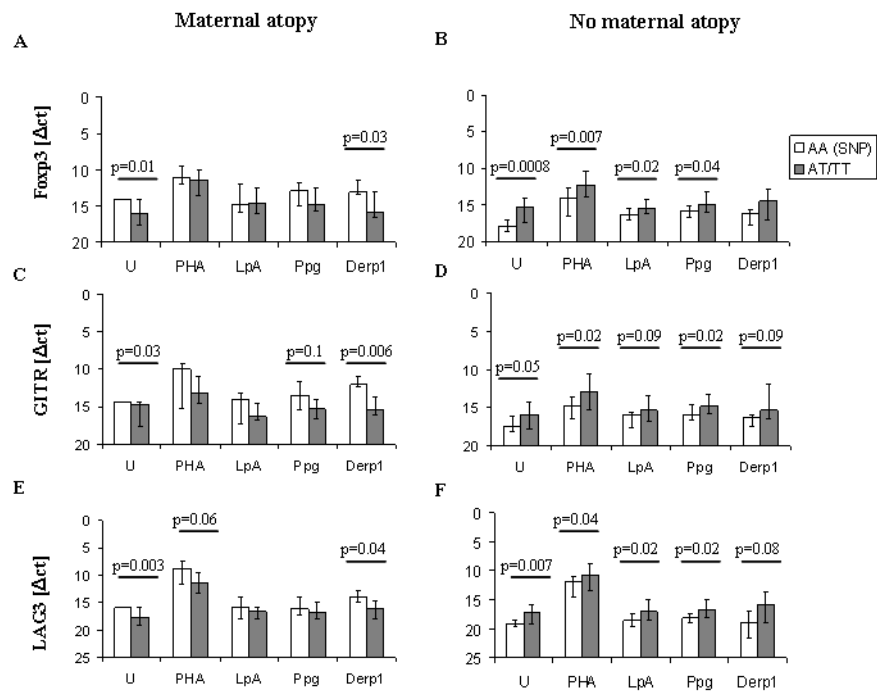


Figure S2 rs1898830 (TLR2)

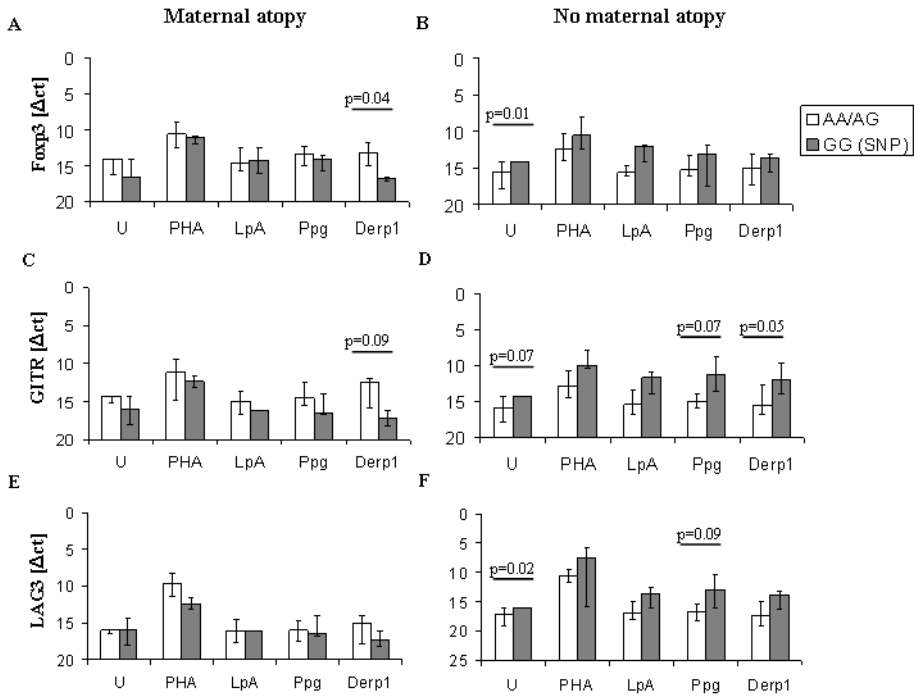


Table S1: mRNA specific primer pairs for RT-PCR of 18S, FOXP3, GITR, LAG3, TLR2

gene	sense primer	anti-sense primer
18S	5'-AGTCCCTGCCCTTTGTACACA-3'	5'-GATCCGAGGGCCTCACTAAAC-3'
FOXP3	5'-ACCTTCCCAAATCCCAGTGC-3'	5'-GAAGATGGTCCGCCTGGC-3'
LAG3	5'-CAATGGCGACTTTACCCTTC-3'	5'-CCTCTGGGATGGGGTGTC-3'
GITR	5'-GCACCACCCTTGTCCTCC-3'	5'-CAGCGTTGTGGGTCTTGTTC-3'
TLR2	5'-TTGGGGGTCATCATCAGCC-3'	5'-GAGCCTGGAGGTTACACACC-3'

Table S2. FOXP3 mRNA expression of rs4833095 (TLR1) and rs4129009 (TLR10) after LpA and Ppg-stimulation

Gene	rs number	Gene_stimulation condition	genotype	n	Fold difference Median (IQR)	p-value*
TLR1	rs4833095	FOXP3_LpA	AA	45	2.91 (1.32-5.28)	0.03
			AG	34	3.4 (1.87-4.92)	
			GG (SNP)	9	0.83 (0.45-2)	
		FOXP3_Ppg	AA	43	3.36 (1.68-6.96)	0.02
			AG	28	4.5 (2.28-6.5)	
			GG (SNP)	9	1.5 (0.93-2.13)	
TLR10	rs4129009	FOXP3_LpA	AA	55	3.14 (1.37-5.46)	0.07
			AG	30	3.18 (1.61-4.56)	
			GG (SNP)	3	0.73 (0.45-0.83)	
		FOXP3_Ppg	AA	52	3.99 (1.74-6.7)	0.07
			AG	25	3.22 (1.74-6.02)	
			GG (SNP)	3	0.93 (0.7-1.5)	

IQR, interquartile range. * Comparison of FOXP3 expression in genetic variants of rs4833095 and rs4129009 (p-value of the Kruskal-Wallis test). FOXP3 expression was shown as fold difference (median $\pm$ IQR).

Table S3: Distribution of genotype frequencies of rs4696480 and rs1898830 (TLR2) in CBMCs with and without maternal atopy

TLR 2	Genotype	Maternal atopy* n (%)	No maternal atopy n (%)	p-value**
rs4696480 (atopy: n = 69; non-atopy: n = 120)	AA (SNP)	27 (39.1)	27 (22.5)	0.03
	TA	31 (44.9)	61 (50.8)	
	TT	11 (15.9)	32 (26.7)	
rs1898830 (atopy: n = 42; non-atopy: n = 67)	AA	24 (57.1)	22 (32.8)	0.04
	AG	14 (33.3)	36 (53.7)	
	GG (SNP)	4 (9.5)	9 (13.4)	

* Maternal atopy was defined as doctor's diagnosis of asthma, allergic rhinitis and/or atopic eczema. **Comparison of genotype frequency between CBMCs of atopic mothers and mothers without atopy (p-value of the Chi2-test).

Table S4: Effect of TLR SNPs on different clinical outcomes

TLR	SNP	Outcome		WT % (n)	HT % (n)	SNP % (n)	p (MH)
TLR1	rs5743595	atopy	0	34 (20)	40 (14)	0 (0)	0.10
			1	66 (39)	60 (21)	100 (6)	
TLR2	rs1898830	wheeze	0	54 (20)	64 (30)	85 (11)	0.07
			1	46 (17)	36 (17)	15 (2)	
TLR2	rs1898830	asthma	0	76 (19)	92 (24)	100 (9)	0.14
			1	24 (6)	8 (2)	0 (0)	
TLR2	rs3804100	asthma	0	85 (74)	100 (12)	0 (0)	0.05
			1	15 (13)	0 (0)	100 (1)	
TLR4	rs4986790	AD ₁	0	86 (77)	62.5 (5)	(x)	0.09
			1	14 (13)	37.5 (3)	(x)	
TLR4	rs4986791	AD ₁	0	88 (29)	50 (2)	(x)	0.06
			1	12 (4)	50 (2)	(x)	
TLR4	rs6478317	food allergy	0	100 (40)	98 (53)	80 (8)	0.0006
			1	0 (0)	2 (1)	20 (2)	
TLR6	rs5743789	AD ₂	0	71 (77)	71 (39)	44 (4)	0.09
			1	29 (31)	29 (16)	56 (5)	

% (n), percentage of children with respective genotype and outcome (number of cases)

0, negative for respective outcome.

1, positive for respective outcome.

$p(MH)$, p value from Mantel-Haenszel test.

x, no SNP genotype in study population.

atopy, doctor's diagnosis or symptoms of atopy.

wheeze, wheeze in the last 36 month.

asthma, doctor's diagnosis of asthma.

AD₁, doctor's diagnosis of atopic dermatitis.

AD₂, atopic dermatitis in the last 36 month (symptoms).

food allergy, doctor's diagnosis of food allergy.

Table S5: Effect of the TLR2 SNPs rs4696480 and rs1898830 on different clinical outcomes stratified for maternal atopy

SNP	atopy*	outcome		WT % (n)		HT % (n)		SNP % (n)		p (MH)
rs1898830	0	wheeze	0	50	(8)	62	(21)	89	(8)	0.07
			1	50	(8)	38	(13)	11	(1)	
	1	wheeze	0	55	(11)	69	(9)	75	(3)	0.51
			1	45	(9)	31	(4)	25	(1)	
rs1898830	0	atopy	0	33	(2)	38	(8)	71	(5)	0.11
			1	67	(4)	62	(13)	29	(2)	
	1	atopy	0	39	(7)	40	(2)	0	(0)	0.29
			1	61	(11)	60	(3)	100	(2)	
rs4696480	0	atopy	0	48	(10)	30	(9)	29	(2)	0.54
			1	52	(11)	70	(21)	71	(5)	
	1	atopy	0	0	(0)	23.5	(4)	43	(9)	0.11
			1	100	(3)	76.5	(13)	57	(12)	
rs4696480	0	food allergy	0	97	(29)	93	(51)	84	(21)	0.10
			1	3	(1)	7	(4)	16	(4)	
	1	food allergy	0	100	(10)	83	(24)	87	(20)	0.90
			1	0	(0)	17	(5)	13	(3)	

% (n), percentage of children with respective genotype and outcome (number of cases)

* *atopy*, maternal atopy.

0, negative for respective outcome.

1, positive for respective outcome.

p(MH), p value from Mantel-Haenszel test.

wheeze, wheeze in the last 36 month.

atopy, doctor's diagnosis or symptoms of atopy.

food allergy, skin manifestation.