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STAT6 polymorphisms are associated with neonatal regulatory T cells and cytokines and atopic diseases at 3 years

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Abstract

Background: The transcription factor *STAT6* is crucial for activation of the interleukin (IL)-4/IL-13 pathway and has been linked to regulatory T cells (Tregs). Associations of *STAT6* polymorphisms with IgE levels were described; however, their impact on neonatal immune responses and early disease development is unknown.

Methods: *STAT6* polymorphisms were genotyped in cord blood mononuclear cells by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Gene expression was assessed by real-time polymerase chain reaction (PCR) and cytokines by Multiplex. At age 3 years, atopic diseases were assessed by questionnaires.

Results: *STAT6* rs324011 but not rs1059513 polymorphism was associated with significant or borderline significant decreased mRNA expression of Treg-associated genes (*FOXP3*, *GITR*, *LAG3*). Heterozygotes and minor allele homozygotes of rs324011 had low levels of tumor necrosis factor alpha (TNF- α) and increased interferon gamma (IFN- γ) ($P \leq 0.04$), while heterozygotes and minor allele homozygotes of rs1059513 had increased TNF- α and Granulocyte-macrophage colony-stimulating factor (GM-CSF) ($P \leq 0.05$). In minor allele homozygotes of rs324011, expression of Treg-associated genes was strongly inverse correlated with IFN- γ (unstimulated, $r = -0.7$, $P = 0.111$; LpA stimulation, $r = -0.8$, $P = 0.011$), but not in heterozygotes or major allele homozygotes. Heterozygotes and minor allele homozygotes of rs324011 presented a lower risk of atopic dermatitis and obstructive bronchitis until age 3 years.

Conclusions: Two *STAT6* polymorphisms were associated with altered immune responses already at birth. *STAT6* rs324011 was associated with lower neonatal Treg and increased Th1 response. Those neonates had a lower risk of atopic dermatitis and obstructive bronchitis until 3 years. Our data suggest a role for *STAT6* polymorphisms in early immune regulation and implications on early atopic disease development.

Abbreviations

AD, atopic dermatitis; CBMCs, cord blood mononuclear cells; CT, cycle threshold; Derp1, *Dermatophagoides pteronyssinus*; FOXP3, forkhead box protein P3; GATA3, GATA-binding protein 3; GITR, glucocorticoid-induced tumor necrosis factor receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HT, heterozygotes; HWE, Hardy-Weinberg equilibrium; IFN- γ , interferon gamma; LAG3, lymphocyte activation gene 3; LpA, lipid A; MAF, minor allele frequency; NF- κ B, nuclear factor 'kappa light chain enhancer' of activated B cells; Ppg, peptidoglycan; RAST, radioallergosorbent test; SNP, single nucleotide polymorphism; *STAT6*, signal transducer and activator of transcription 6; TCR, T-cell receptor; TGF- β 1, transforming growth factor beta1; Treg, regulatory T cell; WT, major allele homozygotes.

The chromosomal region 12q13-q24 has been linked to asthma, atopy, total IgE levels, and allergic rhinitis in several studies (1, 2). The human signal transducer and activator of transcription 6 (*STAT6*) gene maps to this important region (3).

The classical pathway of *STAT6* activation is mediated through binding of interleukin (IL)-4 or IL-13 to their common receptor IL-4R α leading to activation of Janus tyrosine kinases (JAK)1 and JAK3. The activated kinases initiate the phosphorylation and dimerization of *STAT6*, which participates in the activation of IL-4-related pathways (4–7), being crucial in Th2-related immune diseases. The importance of *STAT6* on Th2 cells has been shown in *STAT6* null mice with impaired IL-4-mediated functions, such as Th2 differentiation and IgE class switching (8–11). In a mouse model, Th1 cell differentiation was partly recovered in *STAT6*^{−/−} T-bet^{−/−} CD4⁺ T cells as compared to T-bet^{−/−} CD4⁺ T cells, showing its relevance in inhibiting Th1 differentiation (12). The effect of *STAT6* on regulatory T cells (Tregs), which are responsible for the balance of healthy immune responses, is still controversial. Sanchez-Guajardo et al. (13) showed that in addition to T-cell receptor (TCR)-mediated signals, *STAT6* is required for complete induction of forkhead box protein P3 (*FOXP3*) expression, the major transcription factor of Tregs. In contrast, Chapoval et al. (14) observed that *STAT6* limits both naturally occurring and antigen-induced Tregs. Additionally, *STAT6* was able to inhibit transforming growth factor beta1 (TGF- β 1)-mediated *FOXP3* induction by direct binding to the *FOXP3* promoter (15).

Studies of genetic variants in *STAT6* showed significant associations with regulation of IgE levels in different populations (16–20). Two *STAT6* single nucleotide polymorphisms (SNPs) (rs1059513 and rs324011) were shown to be positively associated with total IgE levels in school-age German children (16).

While modulation of the immune system early in life is crucial for the development of childhood atopic diseases (21–23), it remains unknown whether *STAT6* genetic variants are able to shape immune responses already at birth. Thus, we investigated the role of two relevant IgE-associated *STAT6* SNPs on cord blood immune responses, including Treg and T helper cell gene expression and cytokine responses. We further investigated associations of *STAT6* SNPs on the development of atopic diseases during the follow-up of this birth cohort at age 3 years. The role of maternal atopy was explored in the context of *STAT6* polymorphism-modulated immune responses.

Methods

Study population

This birth cohort consisted of healthy neonates ($n = 200$) born in the area around Munich, Germany. Healthy pregnant mothers were enrolled in the last trimester of pregnancy from July 2005 until September 2007. Exclusion criteria comprised preterm deliveries, multiple gestations (twins, triplets),

perinatal infections, fever around birth, maternal intake of medication, and maternal chronic diseases (24, 25). Samples from cord blood were collected immediately after delivery. Mothers completed detailed questionnaires. Potential covariates such as sex, smoking, birth characteristics, race/ethnicity, siblings, education, previous cesarean section, and miscarriage were noted in the questionnaire and confirmed through medical records. Written informed consent was obtained from all mothers. Ethical approval for the study was obtained from the local human research committee of the Bavarian Ethical Board, LMU Munich, Germany.

Clinical phenotypes

Maternal atopy was defined as doctor's diagnosis of asthma and/or eczema and/or hay fever documented in the personal interview. Total and specific maternal IgE levels were measured by radioallergosorbent test (RAST). A positive specific IgE was defined as 1 or more positive reactions ≥ 0.35 IU/ml to a panel of 20 allergens, which included the most common aeroallergens (mites, *Dermatophagoides pteronyssinus*, and *Dermatophagoides farinae*, birch pollen, hazelnut pollen, timothy grass, mugwort, plantain), animals (cat, horse, dog), *Alternaria*, and food including egg white, milk protein, peanut, hazelnut, carrot, wheat flour, and soy as described (25). 82.6% of atopic mothers had a positive RAST. Nonatopic mothers had no atopic disease and sensitization levels comparable to previous epidemiological studies among nondiseased subjects (26). Childhood clinical outcomes during the first 3 years of life were assessed by questionnaires answered by the parents. Variables were defined as follows: obstructive bronchitis as a doctor's diagnosis of asthma ever or a repeated diagnosis of obstructive bronchitis, food allergy as clinical symptoms or as doctor's diagnosis, atopic dermatitis (AD) as doctor's diagnosis of AD, and wheeze was defined by obstructive symptoms during the last 3 years.

Cell isolation and culture

Cord blood samples were obtained at delivery and processed within 24 h. Cord blood mononuclear cells (CBMCs) were isolated by Ficoll-Hypaque density gradient. Cells were stimulated for 3 days with lipid A (LpA, 0.1 μ g/ml), peptidoglycan (Ppg, 10 μ g/ml), or the allergen house dust mite (Derp1, 30 μ g/ml) and compared with unstimulated cells (media). Cells were harvested for mRNA extraction, and supernatants were used for measurement of cytokines including interferon gamma (IFN- γ), IL-5, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor alpha (TNF- α).

Polymorphism selection and genotyping

For genotyping, DNA was extracted from cord EDTA blood. The selection of the *STAT6* genetic variants for the study was based on previous reports that have shown associations of total IgE levels and *STAT6* rs1059513 and rs324011 in independent study populations (16–18). Furthermore,

haplotype analyses indicated that the observed associations were mainly driven by these two SNPs (16). The genotypes of *STAT6* rs1059513 and rs324011 were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Sequenom Inc., San Diego, CA, USA). SPECTRODESIGNER software (Sequenom Inc.) was used to design polymerase chain reaction assays and associated extension reactions. Amplification and extension reaction conditions were previously described (16).

Quantitative real-time-PCR

Total RNA was isolated with TRI reagent from LpA, Ppg, *Dermatophagoides pteronyssinus* (Derp1)-stimulated CBMCs or unstimulated cells and reverse transcribed into cDNA (Invitrogen, Karlsruhe, Germany). Forward and reverse primers for the genes *18S*rRNA (housekeeping), *FOXP3*, *LAG3*, *GITR*, *STAT6*, *STAT6d*, *STAT6e*, *GATA3*, *TBX21*, *HLX1*, *IL-22*, and *IL-9* genes were designed with Vector NTI advance 10 (Invitrogen). Gene expression was measured by increase in fluorescence caused by the binding of SYBR Green to double-stranded cDNA. Values were normalized by subtracting the corresponding *18S* RNA threshold cycle (Ct) value from the Ct of the gene of interest: $\Delta Ct = ct(\text{gene of interest}) - ct(\text{housekeeping gene})$. Higher ΔCt represents a lower gene expression and vice versa.

Cytokines

Cytokines in the supernatants from stimulated and unstimulated CBMCs were measured with Human Cytokine Multiplex Assay Kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. The limits of detection (pg/ml) were 1.8 (IL-5), 3.0 (TNF- α), 2.1 (IL-13), 1.3 (IFN- γ), and 1.0 (GM-CSF).

Statistical analyses

Cytokine data were generally not normally distributed and could not be transformed to normality; thus, nonparametric Kruskal–Wallis test was applied. For nondetectable cytokine concentrations, the value of 0.01 was assigned for inclusion in the analyses.

Gene expression data were analyzed with the nonparametric Kruskal–Wallis test to compare differences between groups. Values were reported as medians and first and third quartiles. Correlations between gene expression and cytokine secretion were reported as Spearman correlation coefficients. Health outcomes were analyzed with Mantel–Haenszel test. Data were generally reported in 3-group comparisons including the health outcomes in the 3-year follow-up or specified otherwise when using genetic models (recessive or dominant, analyzed with Wilcoxon test). Data were not adjusted for multiple testing as the assessed immunological parameters were highly correlated, and expression of Treg gene markers (*FOXP3*, *GITR* and *LAG3* with each other) and pro-inflammatory and Th2 cytokines were also correlated. Differences were considered significant with $P \leq 0.05$

or as borderline significant with $P \leq 0.1$. In total, 200 children were recruited; however, the number for single analyses varied due to sample availability or nonparticipation in the follow-up at age 3 years. Statistical analyses were performed by SAS (version 9.2; SAS Institute, Cary, NC, USA).

Results

We assessed the influence of two important IgE-associated *STAT6* polymorphisms on immune responses at birth and subsequent development of early atopic diseases in a German birth cohort (population characteristics in Table 1). Genotyping data were available for 191 children. As presented in Table 2, the minor allele frequencies (MAF) in our cohort were comparable to the frequencies described in school-age German children (16). For both polymorphisms, the genotyping success rate was at least 98% and both were in Hardy–Weinberg equilibrium ($P > 0.05$).

STAT6 rs324011 SNP was associated with decreased expression of Treg marker genes at birth

Influence of *STAT6* on Treg cell regulation has been previously shown (13–15). When we assessed mRNA expression of Treg cell-associated genes (*FOXP3*, *GITR*, *LAG3*) in relation to *STAT6* SNPs, no significant differences between groups were observed in relation to *STAT6* rs1059513. Heterozygotes and minor allele homozygotes of rs324011 showed low *LAG3* expression upon Derp1 stimulation ($P = 0.053$, Fig. 1C) (Kruskal–Wallis test, 3-group comparison). Similar patterns of lower Treg marker expression in heterozygotes and minor allele homozygotes were observed for *FOXP3* and *GITR* mRNA expression and the other stimulation

Table 1 Study population characteristics

Parameters	<i>n</i>	<i>N</i>
Female sex, <i>n</i> (%)	89 (46.6)	191
Gestational age, weeks, median (Q1; Q3)	40.0 (39.1; 40.7)	191
Maternal atopic diseases, <i>n</i> (%)	69 (36.1)	191
Asthma, <i>n</i> (%)	13 (6.8)	191
Hay fever, <i>n</i> (%)	50 (26.2)	191
Atopic eczema, <i>n</i> (%)	16 (8.4)	191
Maternal serum total IgE (IU/ml), median (Q1; Q3)	34.6 (13.1; 87.3)	187
Maternal-specific IgE > 0.35 IU/ml, <i>n</i> (%)	107 (57.5)	186
Vaginal delivery <i>n</i> (%)	170 (89.0)	191
Siblings ≥ 1 <i>n</i> (%)	86 (45.0)	191
Clinical outcome up to age 3 years: <i>n</i> (%)		
Doctors diagnosis atopic dermatitis	24 (13.9)	173
Doctors diagnosis food allergy	3 (1.7)	173
Symptoms of food allergy	17 (9.8)	173
Asthma/obstructive bronchitis	22 (12.7)	173
Wheeze	67 (38.7)	173

Q1, 1st quartile; Q3, 3rd quartile.

Table 2 Characteristics of the studied polymorphisms

rs number*	Position in relation to ATG	Position in the gene structure	WT/HT/SNP <i>n</i>	WT/HT/SNP %	MAF (our cohort)	MAF (ISAAC)	<i>P</i> value HWE	Call rate (%)
rs1059513	T12888C	3' UTR	155/33/3	81.15/17.28/1.57	0.10	0.12	0.46	98.56
rs324011	C2892T	Intron 2	74/92/25	38.74/48.17/13.09	0.38	0.37	0.93	98.85

ISAAC, International Study of Asthma and Allergy in Childhood phase II (school-age German children); MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; WT, major allele homozygotes; HT, heterozygotes; SNP, minor allele homozygotes.

Genotyping data were available for 191 children.

*SNP number according to database SNP (<http://www.ncbi.nlm.nih.gov/snp>).

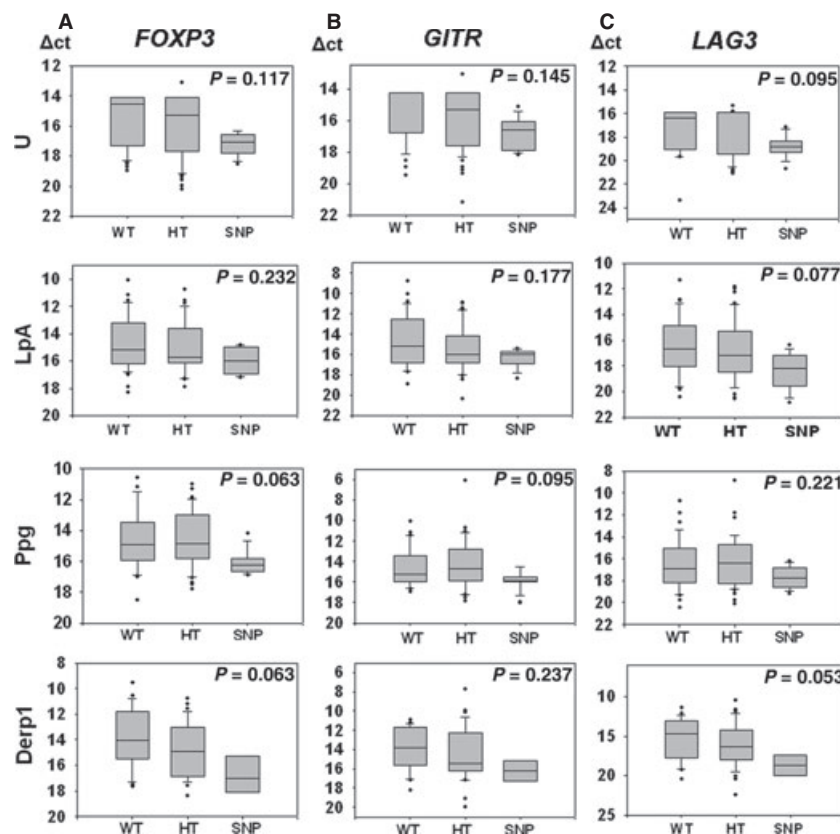


Figure 1 *STAT6* rs324011 allele downregulated Treg cell-related gene expression. (A) *FOXP3*; (B) *GITR*; (C) *LAG3*. Corresponding box plots represent mRNA expression in Δ ct (normalized with *18s*); higher Δ ct represents lower mRNA expression and vice versa. Data were shown as medians, first, and third quartile. WT, major

allele homozygotes; HT, heterozygotes; SNP, minor allele homozygotes; U, unstimulated; LpA, lipid A; Ppg, peptidoglycan; Derp1, *Dermatophagoides pteronyssinus*. Data were analyzed with Kruskal–Wallis test. Maximum number for gene expression analysis: *STAT6* rs324011 *n* (WT) = 43; *n* (HT)=55; *n* (SNP)=10.

conditions. However, this did not reach statistical significance: *FOXP3* (Ppg and Derp1 stimulated, both $P = 0.059$ and 0.063 , Fig. 1A), *GITR* (Ppg, $P = 0.095$, Fig. 1B), and *LAG3* (unstimulated, $P = 0.095$ and LpA $P = 0.077$, Fig. 1C). Applying a recessive model, the differences of lower *FOXP3* (unstimulated, Ppg, Derp1), *GITR* (Ppg), and *LAG3* expression (unstimulated, LpA, Derp1) in minor allele homozygotes became significant ($P \leq 0.05$) (Table S1, Supporting Information). No consistent associations between *STAT6*

polymorphisms and expression of other T helper cell-related genes were found (Data S1).

Influence of *STAT6* polymorphisms on cytokine secretion

Next, we assessed immune responses on a protein level in neonates carrying different *STAT6* genotypes (Table 3). Higher TNF- α production upon LpA and Derp1 stimulation ($P = 0.038/0.052$) and higher GM-CSF production after

Table 3 Cytokine secretion in CBMCs in relation to *STAT6* rs1059513 and rs324011 polymorphisms

Cytokine pg/ml	Stimuli	WT	HT	SNP	P value*
rs1059513					
TNF- α	U $\uparrow$	0.57 (0.01–1.16)	0.55 (0.01–1.35)	0.91 (0.01–9.13)	0.870
	LpA $\uparrow$	943.73 (357.01–1757.48)	1398.86 (423.04–2046.32)	4308.41 (2096.94–7875.38)	0.038
	Ppg $\uparrow$	2254.23 (1302.73–3422.19)	2072.61 (1313.43–3135.92)	6546.62 (2720.25–13075.70)	0.111
	Derp1 $\uparrow$	1024.73 (435.67–1896.18)	1100.52 (547.41–3060.25)	7028.02 (1729.37–13401.76)	0.052
GM-CSF	U $\uparrow$	0.01 (0.01–0.21)	0.01 (0.01–6.36)	2.78 (0.01–14.28)	0.041
	LpA $\uparrow$	2.19 (0.01–14.64)	6.42 (0.01–55.67)	295.54 (13.35–301.23)	0.023
	Ppg $\uparrow$	84.43 (6.07–364.82)	9.72 (0.01–194.80)	112.70 (18.04–781.61)	0.178
	Derp1 $\uparrow$	13.14 (0.01–105.23)	37.24 (1.42–275.90)	623.82 (85.25–649.46)	0.033
IL-13	U $\downarrow$	0.43 (0.24–0.78)	0.37 (0.06–0.84)	0.01 (0.01–0.15)	0.045
	LpA $\uparrow$	4.68 (1.87–12.34)	6.23 (2.01–15.25)	7.75 (7.70–7.92)	0.591
	Ppg $\uparrow$	26.80 (10.48–61.24)	25.39 (9.16–53.74)	27.36 (18.77–34.65)	0.720
	Derp1 $\uparrow$	10.12 (3.40–19.84)	10.22 (3.42–27.26)	18.00 (13.42–20.59)	0.498
IL-5	U $\uparrow\downarrow$	0.10 (0.01–0.17)	0.10 (0.01–0.16)	0.01 (0.01–0.10)	0.441
	LpA $\uparrow$	6.31 (2.64–17.20)	8.23 (3.22–14.60)	9.47 (1.49–47.03)	0.675
	Ppg $\uparrow$	28.94 (11.82–67.65)	25.11 (5.28–44.41)	30.87 (1.79–74.45)	0.514
	Derp1 $\uparrow$	10.73 (4.71–22.59)	9.29 (6.29–22.43)	15.26 (2.70–106.90)	0.833
IFN- γ	U $\uparrow$	0.01 (0.01–1.01)	0.13 (0.01–2.49)	2.53 (0.01–3.18)	0.040
	LpA $\downarrow$	43.84 (6.18–98.95)	24.99 (3.72–76.65)	17.29 (13.78–45.23)	0.514
	Ppg $\downarrow$	49.64 (15.98–130.31)	23.80 (4.96–100.08)	32.95 (25.26–61.60)	0.081
	Derp1 $\downarrow$	47.41 (9.71–121.80)	36.62 (5.63–118.62)	30.56 (13.57–67.71)	0.737
rs324011					
TNF- α	U $\downarrow$	0.77 (0.01–1.24)	0.58 (0.01–1.15)	0.11 (0.01–0.97)	0.161
	LpA $\downarrow$	1124.07 (530.60–1970.03)	938.18 (350.42–1757.48)	787.74 (207.11–1502.44)	0.085
	Ppg $\downarrow$	2589.49 (1403.13–3560.77)	1997.07 (1345.51–3316.99)	1670.61 (1172.47–3144.03)	0.214
	Derp1 $\downarrow$	1121.41 (648.25–2884.49)	1095.66 (395.68–1741.80)	738.75 (346.33–1538.55)	0.044
GM-CSF	U -	0.01 (0.01–2.78)	0.01 (0.01–0.16)	0.01 (0.01–1.02)	0.470
	LpA $\downarrow$	6.07 (0.01–25.20)	1.47 (0.01–20.12)	0.78 (0.01–10.14)	0.484
	Ppg $\uparrow$	61.45 (5.84–219.57)	106.04 (3.64–348.92)	84.94 (9.26–399.79)	0.698
	Derp1 $\downarrow$	34.22 (0.92–208.03)	10.48 (0.01–104.79)	7.60 (0.01–49.87)	0.204
IL-13	U $\uparrow$	0.37 (0.15–0.74)	0.47 (0.27–0.79)	0.41 (0.18–0.79)	0.258
	LpA $\uparrow\downarrow$	4.33 (2.24–14.72)	6.03 (1.93–12.99)	3.11 (1.19–7.80)	0.222
	Ppg $\uparrow\downarrow$	24.39 (11.16–60.86)	28.01 (10.53–65.84)	19.16 (9.32–44.73)	0.528
	Derp1 $\downarrow$	10.46 (5.98–20.59)	10.50 (4.09–28.85)	5.14 (1.99–12.85)	0.063
IL-5	U $\downarrow$	0.13 (0.01–0.19)	0.02 (0.01–0.13)	0.04 (0.01–0.16)	0.069
	LpA $\downarrow$	8.47 (3.66–21.82)	6.25 (2.45–15.35)	4.69 (1.24–8.49)	0.061
	Ppg $\downarrow$	29.13 (10.80–72.92)	32.40 (10.78–68.69)	21.62 (11.82–48.29)	0.567
	Derp1 $\downarrow$	10.97 (6.90–22.59)	11.00 (3.96–26.54)	5.95 (2.94–12.71)	0.059
IFN- γ	U -	0.01 (0.01–1.02)	0.01 (0.01–1.03)	0.01 (0.01–1.02)	0.765
	LpA $\uparrow$	20.90 (3.70–85.07)	48.82 (15.19–105.78)	27.85 (3.86–51.04)	0.020
	Ppg $\uparrow$	27.88 (8.74–117.59)	58.12 (19.30–134.00)	42.51 (7.14–59.11)	0.033
	Derp1 $\uparrow$	23.53 (5.99–119.48)	72.37 (21.79–130.23)	31.87 (5.88–51.69)	0.004

WT, major allele homozygotes; HT, heterozygotes; SNP, minor allele homozygotes; U, unstimulated; LpA, lipid A; Ppg, peptidoglycan; Derp1, *Dermatophagoides pteronyssinus*; CBMCs, cord blood mononuclear cells; $\uparrow$, upregulation or $\downarrow$, downregulation in the presence of the polymorphic allele.

Data presented as medians (first/third quartile).

Significant results are marked in bold.

Maximum number for cytokine analysis: *STAT6* rs1059513 n (WT) = 152; n (HT) = 32; n (SNP) = 3 and *STAT6* rs324011 n (WT) = 73; n (HT) = 90; n (SNP) = 24.

*Kruskal–Wallis test.

LpA and Derp1 stimulation ($P = 0.023/0.033$) were found in minor allele homozygotes of rs1059513. Minor allele homozygotes and heterozygotes had the highest levels of IFN- γ expression in unstimulated cells (3-group comparison,

$P = 0.040$); however, expression was low. After Ppg stimulation, lower IFN- γ secretion was detected, however borderline significant ($P = 0.081$) in the presence of the minor allele.

For the second *STAT6* SNP, minor allele homozygotes and heterozygotes of rs324011 showed significant reduction in TNF- α after Derp1 stimulation ($P = 0.044$) (Table 3) and after LpA stimulation (borderline significant, $P = 0.085$). GM-CSF expression remained unchanged. Regarding Th2 regulation, the main observed effects were borderline significant for lower IL-13 secretion after Derp1 stimulation ($P = 0.063$) and IL-5 after innate (LpA, $P = 0.061$) and allergen stimulation (Derp1, $P = 0.059$). The Th1 hallmark cytokine IFN- γ showed a significant steady pattern of high levels in the minor allele homozygotes and heterozygotes of rs324011 following stimulation of LpA ($P = 0.020$), Ppg ($P = 0.033$), and Derp1 ($P = 0.004$). After applying the recessive model, the majority of data maintained their statistical significance for both polymorphisms (Table S2, Supporting Information). Of note, maternal atopy could not be further investigated due to low numbers of children homozygote for the polymorphisms (Data S1).

Expression of *FOXP3* and *LAG3* was negatively correlated to IFN- γ secretion in minor allele homozygote neonates of *STAT6* rs324011

Next, we assessed correlations between the expression of the master transcription factor of Tregs, *FOXP3*, with IFN- γ (Th1), IL-5, IL-13 (Th2), and TNF- α pro-inflammatory secretion depending on the *STAT6* rs324011 genotypes in unstimulated and stimulated cells (Table 4). In unstimulated cells of major allele homozygotes and heterozygotes, a moderate positive correlation between *FOXP3* expression and IFN- γ ($r = 0.4$, $P = 0.010$; $r = 0.3$, $P = 0.039$, respectively) was observed. In contrast, in minor allele homozygotes, a nonsignificant strong negative correlation ($r = -0.7$, $P = 0.111$) for *FOXP3* and IFN- γ responses was present.

Regarding Th2 and pro-inflammatory cytokines, in major allele homozygotes, significant negative associations between *FOXP3* expression and IL-5 ($r = -0.7$, $P < 0.0001$), IL-13 ($r = -0.4$, $P = 0.006$), and TNF- α ($r = -0.5$, $P = 0.0017$) were shown. A similar pattern was observed for heterozygotes between *FOXP3* expression and IL-5 ($r = -0.4$, $P = 0.001$) and TNF- α ($r = -0.4$, $P = 0.0008$), respectively. Analyses of the total population showed similar correlations as when stratified for heterozygotes and major allele homozygotes (not shown). Like for *FOXP3*, in minor allele homozygotes of rs324011, expression of *LAG3* was also highly inverse correlated with IFN- γ secretion ($r = -0.8$, $P = 0.011$, LpA) (not shown). *GITR* expression was positively correlated with TNF- α ($r = 0.7$, $P = 0.026$) and borderline significant with IL-5 ($r = 0.6$, $P = 0.073$) in the minor allele homozygotes, whereas a significant negative correlation of *GITR* vs IL-5 with TNF- α was observed for the heterozygotes and major allele homozygotes (not shown).

STAT6 rs1059513 major allele homozygotes showed a moderate significant correlation of *FOXP3* with IFN- γ ($r = 0.4$, $P = 0.0001$), IL-5 ($r = -0.5$, $P < 0.0001$), TNF- α ($r = -0.5$, $P \leq 0.0001$), and borderline significant with IL-13 ($r = -0.2$, $P = 0.060$, unstimulated). Minor allele carriers

showed a similar pattern of *FOXP3* correlations with IL-5 and IL-13 ($P \leq 0.05$).

Correlation of *GITR* and *LAG3* expression CBMCs (unstimulated/stimulated) with cytokines depending on rs1059513 was overall consistent with the results for *FOXP3* (not shown).

Presence of *STAT6* rs324011 was associated with a lower risk of atopic dermatitis and obstructive bronchitis during the first 3 years

Finally, we assessed the role of the *STAT6* polymorphisms in the development of early atopic diseases. No associations between *STAT6* rs1059513 and atopy development during the first 3 years were found. However, there were significant associations for *STAT6* rs324011 (Table 5). Major allele homozygotes of rs324011 showed the highest risk of a doctor's diagnosis of AD with 20.6%, while 11.5% of heterozygote children and 4.3% of minor allele homozygotes were diagnosed with AD ($P = 0.046$). In addition, the risk of a doctor's diagnosis of obstructive bronchitis was also the highest in major allele homozygotes (19.1%), while 11.5% of heterozygotes and 0% of minor allele homozygotes were diagnosed with obstructive bronchitis ($P = 0.048$). No significant results were found for parent-reported symptoms of wheeze (not shown), doctor's diagnosis of food allergy, however the number of affected children was rather low (Table 5), nor for parent-reported symptoms of food allergy. When applying the recessive and dominant models (chi-squared test), in relation to the clinical outcomes, the significance of the results before mentioned remained (not shown).

Discussion

The present birth cohort study showed an association of two *STAT6* polymorphisms (rs324011, rs1059513) with immune regulation of T regulatory and Th cell responses at birth, with a subsequent effect on development of atopic diseases with 3 years. Carriers of *STAT6* rs324011 but not rs1059513 polymorphism had decreased Treg-associated gene markers, significantly higher secretion of the Th1 hallmark cytokine, IFN- γ , in parallel with a general decrease in TNF- α . Heterozygote and minor allele homozygote children of rs324011 had a lower risk of a doctor's diagnosis of AD and obstructive bronchitis during the first 3 years of life.

Minor allele homozygotes of rs1059513 had higher levels of TNF- α and GM-CSF, but no effect on atopic outcomes until the age 3 years was observed. Our data indicated no significant differences in the expression of Th1-, Th2-, Th9-, Th17-, or Th22-related genes depending on the genotypes of *STAT6* for either of the two investigated SNPs. Interestingly, the presence of the polymorphic allele of rs324011 was associated with reduced Treg genes exemplified by significantly lower expression of *FOXP3*, *GITR*, and *LAG3* in comparison with major allele homozygotes in both stimulated and unstimulated CBMCs (3-group comparison and recessive model). While the mechanisms of downregulation of Treg genes in *STAT6* rs324011 carriers could not be investigated further in our study due to sample availability, it has been

Table 4 Correlations of *FOXP3* expression with cytokine secretion in CBMCs depending on *STAT6* polymorphisms

Treg marker	Stimuli	Genotype		IFN- γ (pg/ml)	IL-5 (pg/ml)	IL-13(pg/ml)	TNF- α (pg/ml)
rs1059513 <i>FOXP3</i> (Δ Ct)	U	WT	<i>r</i>	0.4	-0.5	-0.2	-0.5
			<i>P</i>	0.0001	<0.0001	0.060	<0.0001
			<i>n</i>	83	84	84	84
		HT + SNP	<i>r</i>	-0.1	-0.5	-0.5	-0.3
			<i>P</i>	0.820	0.022	0.054	0.275
			<i>n</i>	18	18	18	18
	LpA	WT	<i>r</i>	0.1	-0.3	-0.3	-0.1
			<i>P</i>	0.526	0.018	0.011	0.287
			<i>n</i>	72	70	71	72
		HT + SNP	<i>r</i>	0.1	0.2	-0.0	0.1
			<i>P</i>	0.858	0.558	0.979	0.628
			<i>n</i>	13	13	13	13
	Ppg	WT	<i>r</i>	-0.06	0.0	-0.11	-0.2
			<i>P</i>	0.591	0.731	0.365	0.072
			<i>n</i>	66	65	66	66
		HT + SNP	<i>r</i>	-0.3	0.2	0.01	-0.2
			<i>P</i>	0.325	0.415	0.957	0.494
			<i>n</i>	13	13	13	13
	Derp1	WT	<i>r</i>	0.1	-0.3	-0.2	-0.3
			<i>P</i>	0.611	0.061	0.187	0.075
			<i>n</i>	47	46	46	46
		HT + SNP	<i>r</i>	0.4	-0.4	-0.5	-0.7
			<i>P</i>	0.385	0.319	0.233	0.047
			<i>n</i>	8	8	8	8
rs324011 <i>FOXP3</i> (Δ Ct)	U	WT	<i>r</i>	0.4	-0.7	-0.4	-0.5
			<i>P</i>	0.010	<0.0001	0.006	0.0017
			<i>n</i>	41	41	41	41
		HT	<i>r</i>	0.3	-0.4	-0.18	-0.4
			<i>P</i>	0.039	0.001	0.196	0.0008
			<i>n</i>	54	55	55	55
		SNP	<i>r</i>	-0.7	0.3	-0.4	0.4
			<i>P</i>	0.111	0.538	0.351	0.346
			<i>n</i>	7	7	7	7
	LpA	WT	<i>r</i>	-0.1	-0.1	-0.3	-0.2
			<i>P</i>	0.649	0.524	0.097	0.231
			<i>n</i>	36	36	36	36
		HT	<i>r</i>	0.3	-0.24	-0.3	0.0
			<i>P</i>	0.081	0.131	0.047	0.813
			<i>n</i>	43	42	42	43
		SNP	<i>r</i>	0.1	-0.0	-0.3	0.0
			<i>P</i>	0.969	0.957	0.478	0.939
			<i>n</i>	7	6	7	7
	Ppg	WT	<i>r</i>	-0.1	0.3	0.1	-0.0
			<i>P</i>	0.475	0.095	0.471	0.960
			<i>n</i>	32	32	32	32
		HT	<i>r</i>	0.0	-0.1	-0.2	-0.2
			<i>P</i>	0.924	0.642	0.234	0.231
			<i>n</i>	40	40	40	40
		SNP	<i>r</i>	0.14	-0.6	-0.7	-0.3
			<i>P</i>	0.760	0.208	0.071	0.482
			<i>n</i>	6	6	7	7
	Derp1	WT	<i>r</i>	0.3	-0.3	-0.31	-0.3
			<i>P</i>	0.162	0.141	0.164	0.164
			<i>n</i>	21	21	21	21
		HT	<i>r</i>	0.0	-0.1	-0.0	-0.1
			<i>P</i>	0.963	0.585	0.893	0.711
			<i>n</i>	30	29	29	29
		SNP	<i>r</i>	-0.8	-0.4	-0.4	0.0
			<i>P</i>	0.200	0.600	0.600	1.000
			<i>n</i>	4	4	4	4

r, Spearman correlation coefficients; WT, major allele homozygotes; HT, heterozygotes; SNP, minor allele homozygotes. U, unstimulated; LpA, lipid A; Ppg, peptidoglycan; Derp1, *Dermatophagoides pteronyssinus*.

$P \leq 0.01$ is significant.

Heterozygotes and minor allele homozygotes of rs1059513 were combined to increase statistical power.

Table 5 Prevalence of clinical outcomes in children in relation to *STAT6* rs324011 genotypes

Phenotype	Genotypes% (n/N)			P value*
	WT	HT	SNP	
Atopic dermatitis	20.6 (13/63)	11.5 (10/87)	4.4 (1/23)	0.046
Food allergy	1.6 (1/63)	2.3 (2/87)	0.0 (0/23)	0.951
Symptoms of food allergy	11.1 (7/63)	10.3 (9/87)	4.4 (1/23)	0.629
Obstructive bronchitis	19.1 (12/63)	11.5 (10/87)	0 (0/23)	0.048

WT, major allele homozygotes; HT, heterozygotes; SNP, minor allele homozygotes.

Atopic dermatitis = doctor's diagnosis of AD; food allergy = doctor's diagnosis of food allergy; Symptoms of food allergy = parental report of symptoms of food allergy; obstructive bronchitis = doctor's diagnosis of asthma ever or repeated obstructive bronchitis. Outcome data assessed within the group of children with available genotyping data.

Data are shown in percentages and number of cases within the respective groups.

*Differences between groups were calculated with Mantel-Haenszel test.

previously reported that this polymorphism creates a specific binding site for NF- κ B in the *STAT6* gene (27). NF- κ B was shown to play a critical role in the development of Tregs as blockade of the NF- κ B pathway inhibited upregulation of *FOXP3* expression (28) and c-Rel, a NF- κ B family member directly promoted the transcription of *FOXP3* in thymic Tregs (29). One could speculate that more NF- κ B molecules will be bound to the specific binding site created by the rs324011 in the *STAT6* gene, which could in turn decrease the availability of NF- κ B for the Treg-related pathways that might require NF- κ B. Furthermore, rs324011 was shown to upregulate *STAT6* gene expression (27); thus, another possible mechanism could be through direct *STAT6* binding to the *FOXP3* gene, which may lead to reduced *FOXP3* promoter activation (15). However, further functional studies are required.

The changes in Th1, Th2, and pro-inflammatory cytokine responses at protein level in our study depending on the *STAT6* polymorphisms are in accordance with previous reports showing that genetic variants in important asthma/allergy-related genes can modulate cytokine secretion already at birth (21, 22, 30). While *STAT6* regulation can affect further downstream signaling, it may directly or indirectly regulate cytokine production. In this context, we found lower Treg gene expression in parallel to lower pro-inflammatory TNF- α secretion in rs324011 SNP carriers. This cytokine pattern may be indicative of potential 'atopy protection', and Treg induction in order to inhibit pro-inflammatory responses may not be required. In parallel, these neonates presented a Th1-skewed immune response with significantly higher IFN- γ . Furthermore, minor allele homozygotes of rs324011 showed high negative correlations of Treg markers (*LAG3* after LpA stimulation and borderline significant for

FOXP3 in unstimulated cells) with IFN- γ . This relationship was neither observed in the other two groups nor when assessing correlations in all children. In addition, *FOXP3* expression was positively correlated with secretion of IL-5 and TNF- α in the minor allele homozygotes but negatively in the heterozygotes and major allele homozygotes. These findings may reflect a potential combined regulation of Tregs/cytokine profile in cord blood associated with the presence of both polymorphic alleles (rs324011) in the *STAT6* gene.

The other *STAT6* SNP rs1059513 did not show a distinct correlation pattern of Tregs with IFN- γ , and correlation of *FOXP3* with Th2/pro-inflammatory cytokines did not depend on the genotype. This may indicate a more modest role of the rs1059513 variant on the Treg/cytokine balance at birth in comparison with rs324011.

Our data showed a lower risk of early AD and obstructive bronchitis in the presence of the polymorphic allele of rs324011, but no association with rs1059513. Previous studies have reported positive associations between *STAT6* 2964 G/A 3'UTR polymorphism (rs324015) and food allergy (31). Furthermore, *STAT6* polymorphisms were associated with food sensitization in asthmatic Mexican children, including a borderline significance for rs324011 (32); however, in our investigation, no associations with doctor's diagnosis of food allergy were found. Yet, our power may have been limited to detect this, as a maximum of two diagnosed children were present in the heterozygote group and one child in the major allele carrier group; thus, no further conclusions can be drawn. Hence, it may be necessary to address this question in a high-risk cohort.

Other studies have shown that i) rs324011 was associated with higher total IgE levels in childhood and adulthood (16–18) and ii) overall, lower Treg numbers in neonates were associated with the development of egg allergy in early childhood (33, 34). In contrast, our results point to a lower likelihood of AD and obstructive bronchitis until 3 years of age in heterozygotes and minor allele homozygotes of rs324011, which also had lower Treg markers at birth. Potential explanation of the contrary findings of Schedel et al. and Weidinger et al. (16, 18), which showed association of the polymorphic allele rs324011 with increased IgE, to our results may be the difference in clinical phenotype (total IgE vs AD/obstructive bronchitis) and different time of immune maturation (school age and adults vs 3 years). Differences to Smith et al. (33) may be also due to a different phenotype (egg allergy during first year) or difference in Treg assessment.

It is important to note that minor allele homozygotes of rs324011 also had a strong IFN- γ response (and borderline significant low IL-5/13), which might result in a lower propensity for AD and obstructive bronchitis. This is in accordance with other studies showing that lower IFN- γ levels at birth were associated with increased risk of atopic diseases and wheeze (35, 36).

Nonetheless, the continuing follow-up of our cohort is essential as atopic phenotypes are still evolving at this young age. Furthermore, at age 3 years, no lung function test can be performed for a specific asthma diagnosis. Thus, the diagnosis of obstructive bronchitis will be re-evaluated during the

follow-up at the age of 6 years, when a diagnosis of asthma can be objectively made.

Previously, we have shown that maternal atopy can modify polymorphism-associated immune responses (22). In this study, further stratification by maternal atopy was not feasible due to low number of children.

Potential limitations of the study need to be considered. Besides Treg-related genes, it would have been of interest to assess Treg cell number and function *per se*; however, this was not feasible in the entire cohort due to limited cell availability. Cytokine concentrations were measured in the supernatant of CBMCs; thus, the specific cellular source cannot be defined. Statistical power was limited in some analyses due to low number of minor allele homozygote children of the *STAT6* SNPs. Missing effects might potentially be explained by limited power. Of note, we have not adjusted for multiple testing as the assessed parameters are correlated. Treg-associated genes *FOXP3*, *GITR*, and *LAG3* were highly correlated in all stimulation conditions, and the cytokines TNF- α with GM-CSF/IL-5/IL-13, and IL-13 with IL-5 and with GM-CSF, were also positively correlated. Thus, adjustment for multiple testing with Bonferroni would not be valuable for our analysis. The strength of this study is the comprehensive investigation of detailed immune regulation on mRNA and protein level, following innate and allergen stimulation in CBMCs in a well-characterized neonatal population combined with the follow-up until 3 years of age with clinical assessment by questionnaire. This birth cohort study can thus contribute to a better understanding of the genetic effects of *STAT6* polymorphisms on early immune regulation and subsequent development of atopy, but clearly, our findings need to be confirmed in later childhood and in larger cohorts.

In summary, our analyses suggested a role for polymorphisms in the Th2 pathway, particularly for *STAT6* rs324011, on the regulation of immune responses already at an early stage of immune maturation, associated with significantly lower Treg gene expression and Th1 polarization. Carriers of *STAT6* rs324011, having a lower risk of AD and obstructive bronchitis with 3 years, will be followed until the age of 6 years to further investigate atopic and respiratory disease during age-related immune maturation.

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Author contributions

V. Casaca performed data analyses and interpretation and contributed to the writing of the manuscript. S. Illi performed data analyses. E. Klucker performed experiments. N. Ballenberger performed data analyses. M. Schedel, E. v. Mutius, and M. Kabesch contributed to data interpretation and writing of the manuscript. B. Schaub designed the study, performed data analyses and interpretation and wrote the final version of the manuscript.

Conflict of interest

V. Casaca, S. Illi, E. Klucker, N. Ballenberger, and M. Schedel declare no conflict of interests. E. v. Mutius, consultant for Glaxo Smith Kline, Novartis, ProtectImmun, has received funding from DFG and EU. M. Kabesch is a consultant for Sanofi, Speakers Bureau: Roxall, Glaxo Wellcome, Novartis, Allergopharma. M. Kabesch received funding from BMBF, DFG and EU. B. Schaub has received funding from Comprehensive Pneumology Centre, EU, and DFG.

Supporting Information

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

Data S1. *STAT6* polymorphisms are associated with neonatal regulatory T cells and cytokines and atopic diseases at 3 years.

Table S1. Dominant and recessive models comparing results of Treg-associated gene expression depending on *STAT6* rs324011 genotypes.

Table S2. Dominant and recessive models comparing results of cytokine secretion depending on *STAT6* SNP genotypes.

Table S3. Distribution of minor allele frequencies of the *STAT6* polymorphisms in neonates from atopic and non-atopic mothers.

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SUPPORTING INFORMATION

***STAT6* polymorphisms are associated with neonatal Regulatory T cells and cytokines and atopic diseases at 3 years**

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RESULTS

No associations between *STAT6* polymorphisms and expression of other T helper cell-related genes

We further determined the effects of the *STAT6* rs1059513 and rs324011 SNPs on gene expression of Th-related genes in cord blood, including *TBX21* and *HLX1* (Th1), *STAT6* and two *STAT6* isoforms (*STAT6d*, *STAT6e*) and *GATA3* (Th2), *IL-22* (Th17/Th22) and *IL-9*-expression (Th9). When the data were analyzed comparing the three genotypes (Kruskal-Wallis test) no consistent significant differences in gene expression pattern for either of the polymorphisms were found (not shown). Of note, heterozygotes and minor allele homozygotes of rs324011, showed a borderline significant overall downregulation of *STAT6* (e.g after LpA-stimulation, $p=0.175$) and an increase in *HLX1* (U, LpA, Ppg, Derp1) and *TBX21* (LpA) expression was observed, however data were not significant.

STAT6 polymorphisms and maternal atopy

We previously reported on the effect of maternal atopy on SNP-mediated immune responses (1). In this study, both *STAT6* polymorphisms showed a comparable distribution of MAF in neonates with and without maternal atopy (Table S3). Due to low number of children, further stratification by maternal atopy was not possible in this study.

Table S1. Dominant and recessive models comparing results of Treg-associated gene expression depending on *STAT6* rs324011 genotypes.

Parameter Δ ct	Stim.	WT	HT+SNP	P value* Dominant [†]
<i>Foxp3</i>	U	14.52 (14.09-17.30)	16.30 (14.09-17.75)	0.245
	LpA	15.15 (13.45-16.20)	15.70 (14.60-16.30)	0.250
	Ppg	14.90 (13.45-15.90)	15.00 (13.20-16.20)	0.596
	Derp1	14.05 (11.80-15.50)	15.40 (13.10-16.90)	0.095
<i>GITR</i>	U	14.28 (14.28-16.85)	16.05 (14.28-17.63)	0.311
	LpA	15.18 (12.50-16.80)	16.00 (14.90-16.85)	0.121
	Ppg	15.25 (13.48-15.98)	15.05 (13.95-16.00)	0.877
	Derp1	13.80 (11.75-15.50)	15.50 (12.40-16.20)	0.227
<i>LAG3</i>	U	16.36 (15.93-19.05)	17.50 (15.93-19.40)	0.587
	LpA	16.70 (14.90-18.05)	17.65 (15.85-19.05)	0.233
	Ppg	16.92 (15.03-18.15)	16.80 (14.95-18.28)	0.960
	Derp1	14.75 (13.00-17.75)	16.90 (14.43-18.15)	0.100
Parameter Δ ct	Stim.	WT+HT	SNP	P value* Recessive [†]
<i>Foxp3</i>	U	14.95 (14.09-17.40)	17.08 (16.58-17.80)	0.054
	LpA	15.40 (13.43-16.15)	15.98 (15.00-16.93)	0.129
	Ppg	14.88 (13.10-15.83)	16.20 (15.80-16.63)	0.018
	Derp1	14.60 (13.00-16.60)	17.00 (15.30-18.10)	0.050
<i>GITR</i>	U	14.85 (14.28-17.50)	16.63 (16.10-17.90)	0.059
	LpA	15.50 (13.70-16.80)	16.00 (15.75-16.85)	0.156
	Ppg	15.05 (12.95-15.95)	15.88 (15.50-16.00)	0.035
	Derp1	15.00 (12.20-16.20)	16.25 (15.15-17.25)	0.163
<i>LAG3</i>	U	15.93 (15.93-19.13)	18.85 (18.35-19.30)	0.030
	LpA	16.90 (15.08-18.48)	18.20 (17.20-19.50)	0.030
	Ppg	16.75 (14.75-18.20)	17.73 (16.80-18.60)	0.095
	Derp1	15.60 (13.70-17.90)	18.65 (17.40-20.00)	0.041

Data presented as medians (first/third quartile).

*P values calculated with non-parametric Wilcoxon test for group comparison of the medians.

†Dominant model = major allele homozygotes vs heterozygotes and minor allele homozygotes. Recessive model = minor allele homozygotes vs heterozygotes and major allele homozygotes.

WT = major allele homozygotes, HT = heterozygotes, SNP = minor allele homozygotes, U = unstimulated, LpA = Lipid A, Ppg = Peptidoglycan, Derp1 = *Dermatophagoides pteronyssinus*. ↑ = upregulation or ↓ = downregulation in the presence of the polymorphic allele. Maximum number for gene expression analysis: *STAT6* rs324011 n (WT)=43; n (HT)=55; n (SNP)=10. Significant values are marked in bold.

Table S2. Dominant and recessive models comparing results of cytokine secretion depending on *STAT6* SNP genotypes.

Cytok. pg/ml	Stim.	Dominant [†] Model					
		rs1059513			rs324011		
		WT	HT+SNP	P * value	WT	HT+SNP	P * value
TNF-α	U	0.57 (0.01-1.16)	0.55 (0.01-1.47)	0.972	0.77 (0.01-1.24)	0.50 (0.01-1.15)	0.238
	LpA	943.73 (357.01-1757.48)	1555.43 (433.90-2345.46)	0.094	1124.07 (530.60-1970.03)	875.64 (307.17-1721.07)	0.056
	Ppg	2254.23 (1302.73-3422.19)	2112.51 (1330.50-3253.58)	0.975	2589.49 (1403.13-3560.77)	1958.18 (1250.78-3316.99)	0.101
	Derp 1	1024.73 (435.67-1896.18)	1291.52 (688.71-3105.12)	0.114	1121.41 (648.25-2884.49)	976.49 (390.06-1703.11)	0.038
GM-CSF	U	0.01 (0.01-0.21)	0.01 (0.01-6.61)	0.017	0.01 (0.01-2.78)	0.01 (0.01-0.26)	0.545
	LpA	2.19 (0.01-14.64)	8.09 (0.01-162.20)	0.054	6.07 (0.01-25.20)	0.78 (0.01-16.90)	0.243
	Ppg	84.43 (6.07-364.82)	29.03 (1.76-194.80)	0.138	61.45 (5.84-219.57)	98.13 (4.31-391.45)	0.480
	Derp 1	13.14 (0.01-105.23)	41.53 (4.25-276.40)	0.055	34.22 (0.92-208.03)	10.28 (0.01-103.27)	0.083
IL-13	U	0.43 (0.24-0.78)	0.37 (0.01-0.77)	0.183	0.37 (0.15-0.74)	0.47 (0.25-0.79)	0.175
	LpA	4.68 (1.87-12.34)	7.70 (2.24-14.72)	0.348	4.33 (2.24-14.72)	5.21 (1.61-11.62)	0.442
	Ppg	26.80 (10.48-61.24)	25.44 (9.51-50.36)	0.439	24.39 (11.16-60.86)	27.66 (10.42-59.34)	0.714
	Derp 1	10.12 (3.40-19.84)	15.09 (4.09-24.84)	0.382	10.46 (5.98-20.59)	10.17 (3.23-20.92)	0.488
IL-5	U	0.10 (0.01-0.17)	0.10 (0.01-0.16)	0.960	0.13 (0.01-0.19)	0.02 (0.01-0.15)	0.023
	LpA	6.31 (2.64-17.20)	8.24 (3.05-15.37)	0.389	8.47 (3.66-21.82)	5.45 (2.43-14.15)	0.044
	Ppg	28.94 (11.82-67.65)	27.99 (5.28-44.71)	0.250	29.13 (10.80-72.92)	27.69 (11.38-60.34)	0.655
	Derp 1	10.73 (4.71-22.59)	10.13 (6.29-22.43)	0.627	10.97 (6.90-22.59)	9.12 (3.81-22.43)	0.080
IFN-γ	U	0.01 (0.01-1.01)	0.24 (0.01-2.53)	0.017	0.01 (0.01-1.02)	0.01 (0.01-1.03)	0.466
	LpA	43.84 (6.18-98.95)	22.69 (3.74-72.90)	0.253	20.90 (3.70-85.07)	44.23 (9.24-98.31)	0.105
	Ppg	49.64 (15.98-130.31)	24.93 (5.74-61.60)	0.028	27.88 (8.74-117.59)	50.88 (17.40-123.26)	0.073
	Derp 1	47.41 (9.71-121.80)	33.59 (6.89-99.12)	0.440	23.53 (5.99-119.48)	52.53 (13.77-118.44)	0.120
Recessive [†] model							
		rs1059513			rs324011		
		WT+HT	SNP	P value *	WT+HT	SNP	P value *
TNF-α	U	0.55	0.91	0.623	0.65	0.11	0.073

		(0.01-1.17)	(0.01-9.13)		(0.01-1.17)	0.01-0.97	
	LpA	974.66 (369.02-1857.59)	4308.41 (2096.94-7875.38)	0.021	1045.80 (391.87-1934.48)	787.74 (207.11-1502.44)	0.097
	Ppg	2171.48 (1313.43-3313.31)	6546.62 (2720.25-13075.70)	0.045	2270.20 (1359.88-3328.75)	1670.61 (1172.47-3144.03)	0.275
	Derp1	1035.23 (451.42-1977.79)	7028.02 (1729.37-13401.76)	0.027	1103.84 (495.75-2223.45)	738.75 (346.33-1538.55)	0.052
GM-CSF	U	0.01 (0.01-0.87)	2.78 (0.01-14.28)	0.152	0.01 (0.01-0.87)	0.01 (0.01-1.02)	0.408
	LpA	2.62 (0.01-20.12)	295.54 (13.35-301.23)	0.017	4.03 (0.01-20.61)	0.78 (0.01-10.14)	0.527
	Ppg	78.85 (4.86-338.54)	112.70 (18.04-781.61)	0.506	78.85 (4.13-332.10)	84.94 (9.26-399.79)	0.511
	Derp1	15.81 (0.01-107.72)	623.82 (85.25-649.46)	0.025	16.68 (0.01-122.80)	7.60 (0.01-49.87)	0.368
IL-13	U	0.41 (0.21-0.78)	0.01 (0.01-0.15)	0.017	0.40 (0.21-0.77)	0.41 (0.18-0.79)	0.639
	LpA	4.89 (1.87-13.19)	7.75 (7.70-7.92)	0.524	5.21 (1.93-13.29)	3.11 (1.19-7.80)	0.087
	Ppg	25.71 (9.95-60.42)	27.36 (18.77-34.65)	0.991	25.71 (10.53-62.05)	19.16 (9.32-44.73)	0.365
	Derp1	10.12 (3.42-21.83)	18.00 (13.42-20.59)	0.314	10.48 (4.30-24.84)	5.14 (1.99-12.85)	0.019
IL-5	U	0.10 (0.01-0.16)	0.01 (0.01-0.10)	0.225	0.10 (0.01-0.16)	0.04 (0.01-0.16)	0.748
	LpA	6.42 (2.70-15.37)	9.47 (1.49-47.03)	0.679	7.61 (2.89-18.15)	4.69 (1.24-8.49)	0.075
	Ppg	27.69 (11.78-65.77)	30.87 (1.79-74.45)	0.767	31.37 (10.78-71.01)	21.62 (11.82-48.29)	0.292
	Derp1	10.45 (4.84-22.51)	15.26 (2.70-106.90)	0.634	10.99 (5.14-23.12)	5.95 (2.94-12.71)	0.041
IFN-γ	U	0.01 (0.01-1.103)	2.53 (0.01-3.18)	0.149	0.01 (0.01-1.03)	0.01 (0.01-1.02)	0.780
	LpA	40.13 (5.74-96.96)	17.29 (13.78-45.23)	0.662	43.20 (6.40-101.16)	27.85 (3.86-51.04)	0.092
	Ppg	45.49 (12.38-120.71)	32.95 (25.26-61.60)	0.877	46.27 (14.07-127.84)	42.51 (7.14-59.11)	0.209
	Derp1	47.13 (7.95-120.33)	30.56 (13.57-67.71)	0.749	51.00 (10.60-129.22)	31.87 (5.88-51.69)	0.017

Data presented as medians (first/third quartile).

*P values calculated with non-parametric Wilcoxon test for group comparison of the medians.

†Dominant model = major allele homozygotes vs heterozygotes and minor allele homozygotes. Recessive model = minor allele homozygotes vs heterozygotes and major allele homozygotes. *STAT6* rs1059513 n (WT)=152; n (HT)=32; n (SNP)=3 and *STAT6* rs324011 n (WT)=73; n (HT)=90; n (SNP)=24.

WT = major allele homozygotes, HT = heterozygotes, SNP = minor allele homozygotes., U = unstimulated, LpA = Lipid A, Ppg = Peptidoglycan, Derp1 = *Dermatophagoides pteronyssinus*. ↑ = upregulation or ↓ = downregulation in the presence of the polymorphic allele. Significant values are marked in bold.

Table S3. Distribution of minor allele frequencies of the *STAT6* polymorphisms in neonates from atopic and nonatopic mothers.

Polymorphism	Maternal atopy	WT/HT/SNP (n)	MAF	P value*
<i>STAT6</i> rs1059513	No	100/21/1	0.09	0.275
	Yes	55/12/2	0.11	
<i>STAT6</i> rs324011	No	50/55/17	0.36	0.428
	Yes	24/37/8	0.38	

*P value calculated with Mantel-Haenszel test. MAF = minor Allele Frequency. (n) = number of children. WT = major allele homozygotes, HT = heterozygotes, SNP = minor allele homozygotes.

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