

Asthma-associated polymorphisms in 17q21 influence cord blood *ORMDL3* and *GSDMA* gene expression and IL-17 secretion

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Background: In a genome-wide association study, genetic variants on chromosome 17q21 were strongly associated with childhood asthma and orosomucoid 1-like 3 (*ORMDL3*) gene expression. Regulation of the 17q21 locus and its immunologic relevance early in life have not been well characterized.

Objective: We investigated the relation between polymorphisms and mRNA expression of 17q21 locus genes and their influence on T-cell subsets in cord blood.

Methods: In 200 children of our cord blood study, 17q21 polymorphisms were genotyped by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Gene expression was assessed for *ORMDL3*; gasdermin A (*GSDMA*, alias *GSDMI*); gasdermin B (*GSDMB*, alias *GSDML*); Ikaros family zinc finger 3 (*ZNFNIA3*), zona pellucida binding protein 2 (*ZPBP2*); and proteasome (prosome, macropain) 26S subunit, non-ATPase, 3 (*PSMD3*), in cord blood mononuclear cells (CBMCs) and for *ORMDL3* in peripheral blood (real-time RT-PCR). Mononuclear cells were assessed before and after microbial (lipid A/peptidoglycan), phytohemagglutinin, or allergen (Der p 1) stimulation. Regulatory T-associated markers (forkhead box protein 3, glucocorticoid-induced TNF receptor, lymphocyte activation gene 3 mRNA expression) and T_H2/T_H1/T_H17 cytokines were examined.

Results: In CBMCs, single genetic risk variants within 17q21 were associated with increased *ORMDL3* (Der p 1 stimulation; $P \leq .01$) and *GSDMA* expression (phytohemagglutinin/Der p 1 stimulation; $P \leq .05$). Children homozygous for all 4 risk alleles for 17q21 tagging single nucleotide polymorphisms showed increased expression for *ORMDL3* (Der p 1; $P = .002$) and *GSDMA* (phytohemagglutinin; $P = .0009$ /Der p 1; $P = .004$). CBMC *ORMDL3* expression was lower compared with PBMCs ($P \leq .0003$) and increased in both CBMC and PBMC after stimulation (phytohemagglutinin/lipid A/peptidoglycan/Der p 1; $P \leq .006$ and phytohemagglutinin/peptidoglycan; $P < .05$, respectively). No correlation between 17q21 polymorphisms and regulatory T/T_H2/T_H1 lineages was detectable. However, 17q21 risk allele carriers showed significantly increased IL-17 secretion (unstimulated, phytohemagglutinin-stimulated). **Conclusion:** Our results suggest an association of 17q21 polymorphisms with *ORMDL3*, *GSDMA* expression, and IL-17 secretion early in life. These observations may imply a functional role of the 17q21 locus affecting T-cell development during immune maturation. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Chromosome 17q21 locus, cord blood, cytokines, *GSDMA*, IL-17, *ORMDL3*, polymorphism, T-cells

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In a genome-wide association study (GWAS), genetic variants of the chromosome 17q21 locus were strongly associated with childhood asthma.¹ The relation between polymorphisms within the chromosome 17q21 locus and the development of asthma has now been replicated in a number of independent studies including different ethnicities, indicating its relevance as an asthma susceptibility locus.²⁻¹¹

Asthma-associated polymorphisms of 17q21 were shown to alter transcription levels of orosomucoid 1-like 3 (*ORMDL3*) and gasdermin B (*GSDMB*; alias *GSDML*).^{1,10,12,13} In independent cohort studies, transcripts of *ORMDL3* were detectable in a wide range of tissues, including the lung as well as lymphocytes of adults and fetal tissue.^{1,14} *ORMDL3* is a member of a highly conserved protein family (*ORMDL1-3*) anchored in the endoplasmic reticulum as transmembrane protein.¹⁴ *ORMDL3* was recently shown to be involved in Ca²⁺ signaling and unfolded protein response.¹⁵ Further functional studies may contribute to elucidating the underlying mechanisms of *ORMDL3* and its association with asthma.

ORMDL3-associated single nucleotide polymorphisms (SNPs) were suggested to influence mainly childhood-onset asthma.³

Abbreviations used

CBMC:	Cord blood mononuclear cell
CT:	Cycle threshold
GMR:	Geometric mean ratio
GSDMA:	Gasdermin A, alias GSDM1
GSDMB:	Gasdermin B, alias GSDML
GWAS:	Genome-wide association study
LD:	Linkage disequilibrium
LpA:	Lipid A
ORMDL3:	Orosomucoid 1–like 3
PSMD3:	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
SNP:	Single nucleotide polymorphism
ZNFN1A3:	Ikaros family zinc finger 3, alias IKZF3
ZBP2:	Zona pellucida binding protein 2

Neonatal immune development was shown to be relevant for the development of childhood allergic diseases.^{16,17} In this context, *ORMDL3*—as a putative crucial player for asthma—may already be modulated in the maturation of early childhood immune responses. In a birth cohort study, genetic variants within the 17q21 locus were associated with childhood but not adult-onset asthma.^{2,3} This was confirmed by a large consortium-based GWAS of asthma.¹⁸ The investigated SNPs did not confer a risk of eczema, rhinitis, or allergic sensitization.² 17q21 SNPs were associated with early-onset asthma and modulated *ORMDL3* gene expression, putatively affected by rs7216389.¹⁹ Yet the functional role of *ORMDL3* in the development of asthma and its effect on early T-cell differentiation are largely unknown.

The aim of this study was to assess the influence of polymorphisms within the 17q21 locus on mRNA expression of genes within this chromosomal region and T-cell subsets in cord blood. Furthermore, we investigated gene expression of *ORMDL3* in early (cord blood mononuclear cells [CBMCs]) compared with adult life (PBMCs).

METHODS**Study populations**

Fetal cord blood ($n = 200$) was obtained for immunologic analyses and genotyping from neonates born in the Munich Metropolitan area.^{16,17} The subjects were recruited from July 2005 to September 2008 in the last trimester of pregnancy at 2 birth clinics in the Munich area of Germany. Inclusion criteria were healthy neonates and mothers with uncomplicated pregnancies. Exclusion criteria were preterm deliveries, multiple gestations (twins, triplets), perinatal infections, fever around birth, maternal intake of medication, and maternal chronic diseases. Informed consent was obtained from the participating mothers. For comparison with adults, healthy controls (mean $\pm$ SD, 32.8 $\pm$ 8.4 years) were recruited in Munich from the University Children's Hospital Research Center ($n = 18$). The study was approved by the local human research committee of the Bavarian Ethical Board, Ludwig Maximilians University of Munich, Germany.

Isolation and culture of CBMCs and PBMCs

Cord blood mononuclear cells and PBMCs were isolated by density-gradient centrifugation with Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden). CBMCs and PBMCs were stimulated with lipid A (LpA; 0.1 μ g/ μ L), peptidoglycan (10 μ g/ μ L), phytohemagglutinin (5 μ g/ μ L), and *Der-matophagoides pteronyssinus* (Der p 1; 30 μ g/ μ L) for 3 days as previously described^{17,20} and compared with unstimulated samples, which were processed immediately. Cells were collected for mRNA extraction, cytokines were measured in supernatants, and T-cell markers were assessed by RT-PCR.

Quantitative real-time RT-PCR

Total RNA was isolated with TRI Reagent (Invitrogen, Carlsbad, Calif), and reverse transcription of 1 μ g RNA was performed according to the manufacturer's instructions (Qiagen, Hilden, Germany). Specific primers for *ORMDL3*; gasdermin A (*GSDMA*; alias GSDM1); *GSDMB*; IKAROS family zinc finger 3 (*ZNFN1A3*); zona pellucida binding protein 2 (*ZBP2*); proteasome (prosome, macropain) 26S subunit, non-ATPase (*PSMD3*); and the housekeeping gene *18S rRNA* were designed with Vector NTI advance10 (Invitrogen, Karlsruhe, Germany; see this article's Table E1 in the Online Repository at www.jacionline.org). Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green (Applied Biosystems, Foster City, Calif) to double-stranded DNA. The determined cycle threshold (CT) reflects the number of PCR cycles required for the fluorescence signal to exceed the detection threshold, which was set to the log-linear range of the amplification curve. The difference in CT values of 2 genes was used to calculate Δ CT. A higher Δ CT indicates lower mRNA expression.

Cytokine measurements

The concentrations of cytokines in supernatants were measured with Human Cytokine Multiplex Assay Kit according to the manufacturer's instructions (Bio-Rad, Munich, Germany). Detection limits (pg/mL) were 0.8 (IL-5), 2.1 (IL-13), 0.2 (IL-17), and 1.3 (IFN- γ).

Selection of polymorphisms and genotyping

Ten SNPs covering the 17q21 locus associated with the development of asthma in 2 independent study populations of children¹ were genotyped. Genotyping was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom Inc, San Diego, Calif). PCR assays and associated extension reactions were designed with the SpectroDESIGNER software (Sequenom Inc).²¹ Detailed information including specific amplification and extension primers for all genotyping procedures is provided by the authors on request.

Statistical analyses

All SNPs were tested for deviation from Hardy-Weinberg equilibrium by χ^2 test. Linkage disequilibrium (LD) between all SNPs was calculated by using R^2 . The R^2 threshold was set to 0.85 to determine tagging SNPs, each representing 1 bin. Bins correspond to LD blocks. LD patterns in our population were highly comparable to the data reported for the HapMap CEU (Utah residents with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain collection) population (<http://hapmap.ncbi.nlm.nih.gov>). For the combination of tagging SNPs, haplotype frequencies were estimated by using the expectation-maximization algorithm. Because allele frequencies for most of the 17q21 SNPs were close to 50%, the respective risk allele (instead of the minor allele) was reported. The risk allele was defined as the allele increasing the risk for the development of childhood asthma as described in the GWAS publication.¹

Risk and nonrisk haplotypes were defined. The nonrisk haplotype (H1) contains nonrisk alleles only of the 4 tagging SNPs, whereas the risk haplotype (H2) is made up of all respective risk alleles (see this article's Table E2 in the Online Repository at www.jacionline.org). An allele-counting model was assumed for the genotypes (0, homozygous nonrisk; 1, heterozygous; 2, homozygous risk).

Multiple regression analysis and Kruskal-Wallis tests were performed to test for differences of cytokine concentrations for different genotypes. Nondetectable concentrations were set to 0.01 pg/mL to include the subjects in the analysis. Because the distribution of cytokine levels was skewed, variables were log-transformed, and geometric mean ratios (GMRs) and 95% CIs were computed. Validating the results by methods that take censoring into account (generalized Wilcoxon test and Tobit model) resulted in comparable data. All GMRs were reported in an unadjusted model (crude) or in an adjusted model for maternal atopy. Statistical significance was defined by $P < .05$.

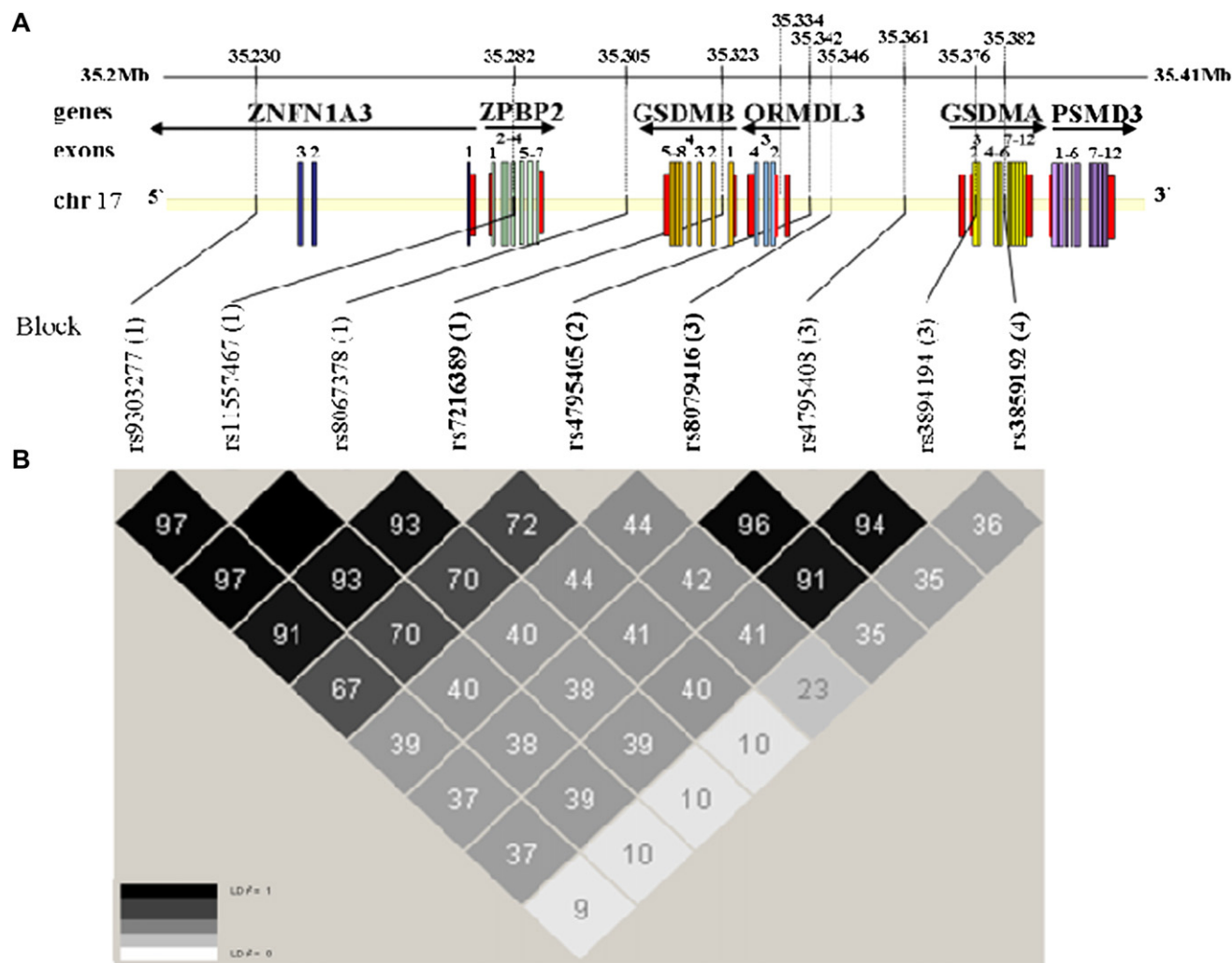


FIG 1. Genomic structure, localization, and linkage disequilibrium (R^2 plot) of the genotyped polymorphisms in the 17q21 locus. Arrows indicate transcriptional direction. Exons with exon numbers are displayed in color codes. Smaller red boxes represent 5' and 3' untranslated regions. Tagging SNPs ($R^2 > 0.85$) for each linkage disequilibrium block (brackets) are depicted in boldface. Chr, Chromosome; Mb, Megabase.

For RT-PCR analysis, a Wilcoxon or signed-rank test was used for the comparison of ΔCT values because some ΔCT values were not normally distributed. A Bonferroni-based correction was used to adjust for multiple testing. As we tested gene expression in relation to 4 tagging SNPs after no stimulation and innate and adaptive stimulation ($n = 3$), we used a significance threshold of $.05/12 = .004$ for SNP analyses. Data below the multiple testing significance threshold of $P \leq .004$ were marked (*) in all figures. Data analyses were performed with SigmaStat3.5 (Jandel Scientific, San Rafael, Calif) and the SAS statistical software package (version 9.2; SAS, Inc, Cary, NC).

RESULTS

Risk alleles of SNPs within the 17q21 locus were associated with increased *ORMDL3* and *GSDMA* gene expression after stimulation

We assessed the genotype of 10 SNPs within the 17q21 locus previously shown to be relevant for childhood asthma (Fig 1; Table I). The genotyping success rate was at least 94%, whereas

rs2290400 (block 1) was excluded from all analyses because genotyping was not successful. None of the polymorphisms significantly deviated from Hardy-Weinberg equilibrium (Table I). On the basis of LD analyses, 4 major LD blocks (Fig 1) were identified, each represented by a tagging SNP (rs7216389, rs4795405, rs8079416, rs3859192).

First we evaluated whether the genotype status of 17q21 polymorphisms might have an influence on *ORMDL3* gene expression already early in life. The respective risk allele was defined as the allele increasing the risk for development of childhood asthma corresponding to GWAS.¹ Hence, gene expression was compared between homozygous carriers of the risk alleles and homozygous carriers of the nonrisk alleles for the 4 investigated tagging SNPs (Fig 2, block 1; see this article's Fig E1, blocks 2-4, in the Online Repository at www.jacionline.org). Gene expression was shown as CT (axis vice versa), and lower CT values corresponded to higher gene expression. Homozygous carriers of the risk alleles for rs7216389 (block 1) showed an increase of *ORMDL3* gene expression compared with homozygous

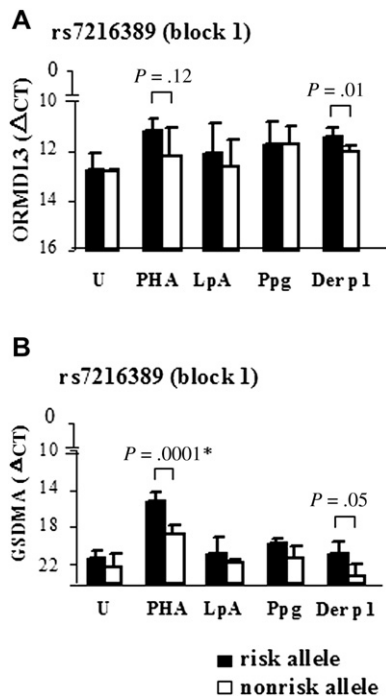
TABLE I. Genotyping information for polymorphisms within the Chr17q21 locus (N = 200)

Genotyped SNP	Nonrisk allele	Risk allele	AF* risk allele	Call rate (%)	LD blocks for 17q21	P value (HWE†)
rs9303277	T	C	0.49	95.0	Block 1	.99
rs11557467	T	G	0.48	94.5	Block 1	.96
rs8067378	G	A	0.48	94.0	Block 1	.90
rs2290400‡					Block 1	
rs7216389	C	T	0.48	95.5	Block 1	.70
rs4795405	T	C	0.55	94.0	Block 2	.58
rs8079416	T	C	0.45	95.0	Block 3	.99
rs4795408	G	A	0.44	95.5	Block 3	.88
rs3894194	G	A	0.44	95.5	Block 3	.99
rs3859192	C	T	0.44	95.5	Block 4	.19

AF, Allele frequency.

Boldface represents the identified tagging SNPs for the 17q21 locus based on $R^2 > 0.85$.*Because AFs for most of the 17q21 SNPs were close to 50%, the respective risk allele (instead of the minor allele) is depicted. This was defined as the allele increasing the risk for the development of asthma as shown in previous publications.¹†P value of χ^2 test for deviation of Hardy-Weinberg equilibrium.

‡Genotyping failed for rs2290400.

**FIG 2.** *ORMDL3* and *GSDMA* gene expression was increased in carriers of 17q21 risk alleles (black bars) in comparison with nonrisk alleles (white bars) after Der p 1 stimulation in CBMCs. **A** and **B**, rs7216389, n = 13/11. Δ CT values are shown; axes are vice versa; lower Δ CT values between groups correspond to increased mRNA expression. Data are shown as median ($\pm$ interquartile range); P values are calculated by the Wilcoxon test. Gene expression on mRNA levels was assessed in subjects homozygous for the risk or nonrisk alleles. PHA, Phytohemagglutinin; Ppg, peptidoglycan; U, unstimulated. *Significance of $P \leq .004$ (below the multiple testing significance threshold).

carriers of the respective nonrisk alleles (Fig 2, A). These differences were significant for Der p 1-activated *ORMDL3* expression ($P = .01$). In parallel, Der p 1-induced and phytohemagglutinin-induced *GSDMA* gene expression was also increased ($P \leq .05$). Sequencing of the PCR product confirmed

specific amplification of *GSDMA* (not shown). Comparable effects for *ORMDL3* and *GSDMA* expression were observed for 17q21 tagging SNPs from blocks 2 to 4 (Fig E1; see this article's Results in the Online Repository at www.jacionline.org).

Results remaining significant after adjustment for multiple testing were marked (*). All effects were independent of maternal atopy (data not shown). Furthermore, other genes of the 17q21 locus, namely *GSDMB*, *ZNFNIA3*, *ZPBP2*, and *PSMD3*, were analyzed. For rs3859192, a trend to lower expression of *ZNFNIA3* (peptidoglycan-stimulated) was observed within the risk allele carriers ($P = .07$; not shown).

Children homozygous for risk alleles for all tagging SNPs of 17q21 showed a cis-regulatory effect on *ORMDL3* and *GSDMA* mRNA expression after stimulation

Haplotype frequencies were estimated on the basis of the four 17q21 tagging SNPs. Two haplotypes were observed that contained either only nonrisk alleles of 17q21 (H1 frequency, 0.36; Table E2) or the combination of the respective risk alleles (H2 frequency, 0.29). Because (1) LD is strong in the 17q21 region and (2) risk and nonrisk alleles were almost equally distributed, it was possible to select a number of children that were either homozygous for the risk alleles at all 4 SNP loci (n = 9) and to compare these with homozygous carriers of the nonrisk alleles (n = 8) at the respective positions (diplotypes).

Children homozygous for all 4 risk alleles for 17q21 tagging SNPs showed increased expression for *ORMDL3* (Der p 1) and *GSDMA* (phytohemagglutinin/Der p 1); both remained significant after adjustment for multiple testing (*; Fig 3, A and B). This was in accordance with the reported effects for individual risk alleles because the respective polymorphisms were not completely independent (Fig 2; Fig E1; Table E2).

Furthermore, it was assessed whether the combination of only 17q21 risk alleles also influenced mRNA expression of other genes located within the 17q21 cluster including *GSDMB*, *ZNFNIA3*, *ZPBP2*, and *PSMD3*. When homozygous carriers of the four 17q21 risk alleles and carriers of nonrisk alleles were compared, a trend to decreased expression of *ZNFNIA3* after peptidoglycan stimulation was detectable (Fig 3, D). No significant effect on gene expression for additionally analyzed genes of 17q21 (*GSDMB*, *ZPBP2*, *PSMD3*) was observed (Fig 3, C, E, F).

Polymorphisms in the 17q21 cluster were associated with changes in IL-17 secretion in unstimulated and stimulated cells

Furthermore, we hypothesized that polymorphisms in the 17q21 cluster might influence T-cell cytokine secretion. None of the presented tagging SNPs influenced T_H1 (IFN- γ) or T_H2 (IL-13, IL-5) cytokine secretion, or regulatory T-cell markers (forkhead box protein 3, glucocorticoid-induced TNF receptor, lymphocyte activation gene 3 mRNA expression; data not shown).

However, homozygous carriers of the risk allele of each tagging SNP individually within the 17q21 locus (rs7216389, rs4795405, rs8079416, rs3859192) showed increased IL-17 secretion in unstimulated and phytohemagglutinin (Table II) but not innate (LpA, peptidoglycan) stimulated cord blood samples (data not shown). When polymorphisms within 1 LD block were analyzed separately, similar results were observed for

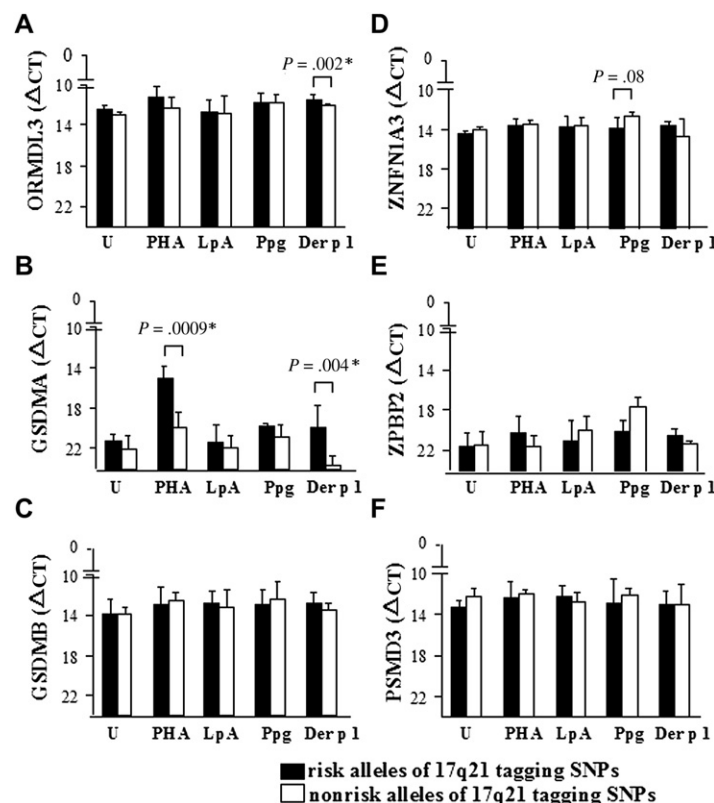


FIG 3. *ORMDL3* and *GSDMA* gene expression was increased in homozygous carriers of the risk haplotype (black bars) in comparison with the nonrisk haplotype (white bars) after Der p 1 and/or phytohemagglutinin (PHA) stimulation in CBMCs. mRNA expression of genes located in 17q21 locus ($n = 9/8$). Δ CT values are shown; axes are vice versa; lower Δ CT values between groups correspond to increased mRNA expression. Data are shown as median ($\pm$ interquartile range), analyzed applying the Wilcoxon test. mRNA expression was assessed in subjects homozygous for the risk or the nonrisk haplotype. Ppg, Peptidoglycan; U, unstimulated. *Significance of $P \leq .004$ (below the multiple testing significance threshold).

each SNP (see this article's Table E3, A and B, in the Online Repository at www.jacionline.org). IL-17 secretion was detectable but low. Both parametric and nonparametric tests showed significance for the same associations. In subjects homozygous for all four 17q21 risk alleles, a trend was observed comparable to that for individual SNP analyses, yet it was not significant ($P < .1$). This may potentially be a result of the lower number of samples (see this article's Table E4 in the Online Repository at www.jacionline.org). To address the question of whether SNPs on the 17q21 locus may have an effect on IL-17 secretion primarily for the children with risk for atopy, GMRs were adjusted for maternal atopy. Yet no significant influence of maternal atopy on the association of IL-17 and the respective genotype was observed in cord blood (Table II; Table E3, A and B).

***ORMDL3* gene expression was lower in neonates (CBMCs) compared with adults (PBMcs)**

Because *ORMDL3* expression was strongly regulated by genetic factors, we further assessed whether *ORMDL3* expression may also depend on the state of immune maturation. In CBMCs, *ORMDL3* mRNA expression was lower compared with PBMcs (Fig 4, A). CBMC *ORMDL3* expression was significantly increased after stimulation with phytohemagglutinin, LpA, peptidoglycan, and Der p 1 (all $P \leq .006$; Fig 4, B). In PBMcs, *ORMDL3*

mRNA expression was significantly increased after phytohemagglutinin and peptidoglycan stimulation, respectively ($P \leq .04$; Fig 4, C). Expression of *ORMDL3* in CBMCs was not significantly different when data were stratified for maternal atopy (not shown). However, the sample size was low ($n = 12$ vs 13).

DISCUSSION

The 17q21 chromosomal region is a well replicated susceptibility locus for the development of asthma, especially for childhood-onset asthma.^{1-11,18} Yet little is known about how this locus influences asthma pathogenesis early in life. In our study, 17q21 polymorphisms were strongly associated with *ORMDL3* and *GSDMA* gene expression already in cord blood. In addition, 17q21 SNPs influenced IL-17 secretion in CBMCs, pointing to a functional role of the 17q21 locus for T-cell regulation early in life. Furthermore, *ORMDL3* mRNA expression was significantly lower in CBMCs compared with PBMcs and significantly increased in CBMCs after stimulation (innate and adaptive).

Thus, we demonstrated that the asthma risk alleles from the 17q21 locus correlate with *ORMDL3* gene expression not only in immortalized lymphocytes later in life¹ but also in cord blood, primarily after Der p 1 stimulation. However, this finding needs to be interpreted with caution because *in vitro* data cannot be transferred directly into *in vivo* situations. One possible explanation

TABLE II. Risk alleles of 17q21 tagging SNPs increased IL-17 secretion in unstimulated and phytohemagglutinin-stimulated cord blood (maximum, n = 200)

Tagging SNP	Stimulation condition of IL-17	Genotype	n	Geometric mean (95% CI)	GMR (95% CI) P value†	
					Crude	Adjusted for maternal atopy
rs7216389 (Block 1)	U	TT risk allele*	43	0.19 (0.11-0.35)	1.83 (1.22-2.75) P = .004	1.83 (1.22-2.76) P = .004
		CT	96	0.10 (0.07-0.15)		
		CC	48	0.06 (0.03-0.10)		
	PHA	TT risk allele*	40	0.26 (0.12-0.56)	1.67 (1.04-2.66) P = .03	1.67 (1.04-2.67) P = .03
		CT	84	0.10 (0.06-0.16)		
rs4795405 (Block 2)	U	CC risk allele*	54	0.15 (0.09-0.26)	1.73 (1.14-2.62) P = .01	1.73 (1.14-2.62) P = .01
		CT	96	0.11 (0.08-0.17)		
		TT	35	0.05 (0.02-0.10)		
	PHA	CC risk allele*	48	0.25 (0.12-0.49)	1.79 (1.11-2.90) P = .02	1.79 (1.11-2.91) P = .02
		CT	86	0.11 (0.07-0.17)		
rs8079416 (Block 3)	U	CC risk allele*	38	0.23 (0.12-0.44)	1.73 (1.15-2.59) P = .009	1.72 (1.15-2.59) P = .009
		CT	92	0.09 (0.06-0.14)		
		TT	56	0.07 (0.04-0.13)		
	PHA	CC risk allele*	35	0.25 (0.11-0.55)	1.66 (1.04-2.64) P = .04	1.66 (1.04-2.64) P = .04
		CT	81	0.12 (0.08-0.20)		
rs3859192 (Block 4)	U	TT risk allele*	41	0.20 (0.11-0.37)	1.52 (1.03-2.25) P = .04	1.53 (1.03-2.26) P = .04
		CT	84	0.09 (0.06-0.13)		
		CC	62	0.08 (0.05-0.13)		
	PHA	TT risk allele*	35	0.23 (0.11-0.51)	1.42 (0.90-2.25) P = .14	1.42 (0.90-2.26) P = .14
		CT	77	0.10 (0.06-0.17)		
		CC	57	0.11 (0.06-0.19)		

PHA, Phytohemagglutinin-stimulated; U, unstimulated.

*The respective risk allele was defined as the allele increasing the risk for the development of asthma as shown in the original publication.¹

†P value of GMR by regression models using an allele-counting model (0, 1, 2) for coding of the respective SNP.

is that carriers of risk alleles may have a greater susceptibility to allergen sensitization. Interestingly, not only *ORMDL3* but also *GSDMA* mRNA expression was affected by the allelic state of the 17q21 SNPs.

Our findings of increased *GSDMA* expression (after phytohemagglutinin and Der p 1 stimulation) in subjects carrying all 4 risk alleles of 17q21 tagging SNPs are novel. Previous studies have described a correlation between genetic variations within this region and regulation of *ORMDL3* and *GSDMB* gene expression.^{10,12,13,19} Both Verlaan et al¹⁰ and Halapi et al¹⁹ observed a correlation of an asthma-associated haplotype with *GSDMB* and *ORMDL3* expression and in the first study also with lower *ZPBP2* expression. These previous findings were suggested to be attributable to allele-specific chromatin remodeling within the 17q12-q21 region.¹⁰ In our analyses, we not only measured expression of the previously analyzed 4 candidate genes

(*ORMDL3*, *GSDMB*, *ZNFN1A3*, *ZPBP2*)¹⁰ but also included *GSDMA* and *PSMD3* located within this disease-associated locus. In addition, *ZNFN1A3* mRNA expression was influenced by genetic variants yet reached only borderline significance after microbial peptidoglycan stimulation. In contrast with the findings of Verlaan et al¹⁰ and others,¹⁹ *ZPBP2* and *GSDMB* gene expression levels were unaffected by the respective genotype status in our population. Whether the differences among studies are a result of different cell populations or the time of assessment because we studied gene expression very early in life cannot be distinguished. Taking our and previously published results into consideration, polymorphisms within 17q21 seem to regulate not only *ORMDL3* but also additional genes such as *GSDMA* and/or *GSDMB*.

In addition to increased *ORMDL3* and *GSDMA* gene expression, IL-17 protein expression was elevated in carriers of the

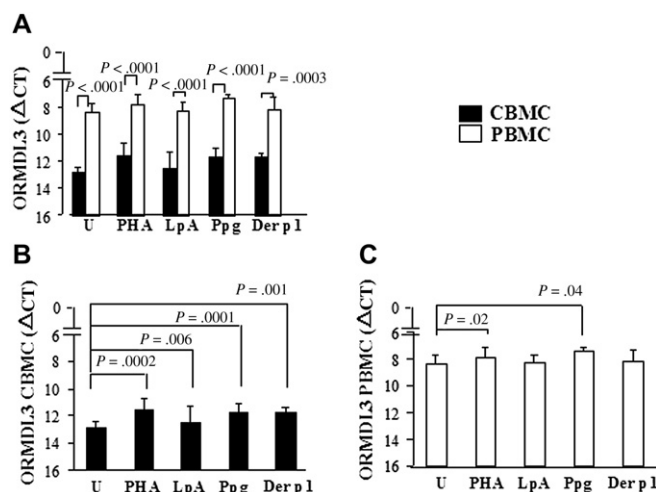


FIG 4. *ORMDL3* expression in CBMCs and PBMCs. mRNA expression of *ORMDL3* was assessed in CBMCs ($n = 25$) and PBMCs ($n = 18$) by real-time RT-PCR. Data are shown as median ($\pm$ interquartile range), analyzed applying the Wilcoxon (A) or the signed rank test (B and C); axes are vice versa. PHA, Phytohemagglutinin; Ppg, peptidoglycan; U, unstimulated.

asthma risk alleles within the 17q21 locus. IL-17 protein expression *per se* was low but detectable in our study population and in accordance with previously published data.¹⁷ In contrast, 17q21 polymorphisms had no effect on other T-cell lineage markers including T_H1 , T_H2 , and regulatory T cells. Thus, T_H17 cells, a cell population suggested to be relevant for asthma development,^{22,23} may be influenced by genetic variations within the 17q21 locus. Our data are a first indication for this association, yet the underlying mechanisms are not clear.

Although the study size is small compared with large population genetic studies used in GWASs, the strength of this study is the detailed immunologic evaluation of study subjects very early in life, when the immune system is vulnerable for the development of allergic diseases.

The observed differences of *ORMDL3* mRNA expression in cord compared with adult blood and the reports on the relevance of *ORMDL3* primarily in childhood asthma are intriguing.^{11,18,19} This may potentially indicate a role for *ORMDL3* during early immune maturation.

However, data on the role of *ORMDL3* and other 17q21 genes in early childhood are still missing, and the underlying mechanism for how genetic variations in these genes contribute to childhood asthma development is currently unknown. According to the results of this study, a focus for further investigations should be on the effects of *ORMDL3* and *GSDMA* expression in early asthma development. For *ORMDL3*, a role in Ca^{2+} signaling was described,¹⁵ which itself is involved in downstream events such as effector T-cell regulation.^{24,25} Furthermore, Orosomucoid family proteins were shown to be relevant for the metabolism of sphingolipids in *Saccharomyces cerevisiae*.²⁶ How that may relate to the development of inflammatory processes such as in asthma needs to be investigated in more detail.

Taken together, our results indicate that carriers of asthma risk alleles within the 17q21 locus showed increased *ORMDL3* and *GSDMA* gene expression and elevated IL-17 secretion in cord blood. Possibly genes of the 17q21 locus may be involved in T-cell effector function. Expression of *ORMDL3* and other genes

of the 17q21 locus was already detectable early in life and induced by innate and adaptive stimuli. Further studies are required to examine whether 17q21 SNP-related differences in T-cell function during immune maturation could subsequently influence the development of childhood asthma.

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Key messages

- Genetic variants within the well replicated asthma-associated region of chromosome 17q21 were associated with altered expression levels of *ORMDL3* and *GSDMA* and IL-17 secretion in this cord blood study.
- These allele-specific effects of 17q21 polymorphisms on T-cell subsets may reveal a novel mechanism critical for the maturing immune system in the development of immune-mediated diseases.

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RESULTS

Individual risk alleles of SNPs within the 17q21 locus were associated with increased *ORMDL3* and *GSDMA* gene expression after stimulation

The homozygous carriers of the risk alleles for each tagging SNP showed an increase of *ORMDL3* gene expression compared with homozygous carriers of the respective nonrisk alleles. Because 17q21 tagging SNPs are not independent of each other, no homozygous carriers of the risk alleles for each tagging SNP alone were available for analysis. The differences were significant for Der p 1-activated *ORMDL3* expression (Fig E1, A-C;

all $P \leq .01$). Comparable effects of tagging SNPs on *GSDMA* mRNA expression after Der p 1 stimulation ($P \leq .05$) and phytohemagglutinin stimulation ($P \leq .001$) were observed (Fig E1, D-F). In addition, a trend for a genotype-dependent effect on *GSDMA*-expression was detectable for rs4795405 after peptidoglycan stimulation (Fig E1, D). Most results remained significant after adjustment for multiple testing (*).

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- E1. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating *ORMDL3* expression contribute to the risk of childhood asthma. *Nature* 2007;448:470-3.

TABLE E1. List of primers used for real-time RT-PCR

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
18SrRNA	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
ZNFN1A3	CCCAGCCAATGAAGATGAAGAC	GCCAACCAGTGGAAGATGAAC
ZBP2	GATTTTTTGGAGCCTTTGTCTGG	CAATGGATCTGTTGACTGTGAAGATAC
GSDMA	GAAGGTGAAGGGAACGGCAG	GTCACACTGGAGCGAGCCG
ORMDL3	GGACCAGGGCAAGGCGAG	CCGTGTCCTGATGAGCGTG
GSDMB	TTTCAGGCAGTTTCCAGGGC	GAAACCCTGAAAAGCGACCG
PSMD3	GCCAGCATCAACCACGAGAAG	GGCGTGAGCGAGAACAGCA

TABLE E2. Estimated haplotypes on the basis of the 4 tagging SNPs within the chromosome 17q21 region and their frequencies (n = 187 with complete phase information)

Haplotype	rs7216389	rs4795405	rs8079416	rs3859192	Estimated frequency
H1	C	T	T	C	0.36
H2	T	C	C	T	0.29
H3	T	C	T	C	0.09
H5	T	C	C	C	0.09
H5	C	T	T	T	0.05
H6	C	C	T	T	0.05
H7	C	T	C	T	0.03
H8	C	C	C	T	0.02
H9	C	C	C	C	0.007
H10	T	T	T	C	0.007
H11	C	T	C	C	0.004
H12	T	T	T	T	0.002

H1 represents the nonrisk and H2 the risk haplotype.

TABLE E3. Risk alleles of 17q21 SNPs increased IL-17 secretion in unstimulated and phytohemagglutinin-stimulated cord blood (N = 200)

A, Block 1					
Cytokine	Genotype	n	Geometric mean (95% CI)	GMR (95% CI) P value†	
				Crude	Adjusted for maternal atopy
rs9303277					
IL-17 U	CC risk allele*	45	0.18 (0.10-0.33)	1.64 (1.09-2.46) P = .018	1.65 (1.10-2.47) P = .017
	CT	93	0.10 (0.07-0.15)		
	TT	48	0.07 (0.04-0.12)		
IL-17 PHA	CC risk allele*	41	0.26 (0.12-0.55)	1.61 (1.01-2.57) P = .048	1.61(1.01-2.59) P = .048
	CT	83	0.10 (0.07-0.16)		
	TT	44	0.10 (0.05-0.19)		
rs11557467					
IL-17 U	GG risk allele*	44	0.19 (0.11-0.35)	1.73 (1.15-2.59) P = .009	1.74 (1.16-2.60) P = .008
	GT	92	0.10 (0.07-0.15)		
	TT	49	0.06 (0.04-0.12)		
IL-17 PHA	GG risk allele*	40	0.28 (0.13-0.59)	1.71 (1.07-2.73) P = .026	1.72 (1.07-2.75) P = .025
	GT	82	0.11 (0.07-0.17)		
	TT	45	0.09 (0.05-0.18)		
rs8067378					
IL-17 U	AA risk allele*	44	0.19 (0.11-0.35)	1.73 (1.15-2.59) P = .009	1.74 (1.16-2.60) P = .008
	AG	92	0.10 (0.07-0.15)		
	GG	49	0.06 (0.04-0.12)		
IL-17 PHA	AA risk allele*	40	0.28 (0.13-0.59)	1.71 (1.07-2.73) P = .026	1.72 (1.07-2.75) P = .025
	AG	82	0.11 (0.07-0.17)		
	GG	45	0.09 (0.05-0.18)		
B, Block 2					
Cytokine	Genotype	n	Geometric mean (95% CI)	GMR (95% CI) P value†	
				Crude	Adjusted for maternal atopy
rs4795408					
IL-17 U	AA risk allele*	36	0.22 (0.11-0.43)	1.67 (1.11-2.51) P = .016	1.67 (1.11-2.51) P = .016
	AG	93	0.09 (0.06-0.14)		
	GG	58	0.07 (0.04-0.13)		
IL-17 PHA	AA risk allele*	33	0.27 (0.12-0.61)	1.60 (1.00-2.56) P = .052	1.60 (1.00-2.57) P = .053
	AG	82	0.11 (0.07-0.17)		
	GG	54	0.10 (0.05-0.17)		
rs3894194					
IL-17 U	AA risk allele*	37	0.23 (0.12-0.43)	1.61 (1.07-2.42) P = .023	1.62 (1.07-2.43) P = .023
	AG	92	0.08 (0.06-0.13)		
	GG	58	0.08 (0.05-0.13)		
IL-17 PHA	AA risk allele*	34	0.24 (0.11-0.56)	1.51 (0.94-2.41) P = .091	1.51 (0.94-2.42) P = .091
	AG	82	0.11 (0.07-0.17)		
	GG	53	0.10 (0.05-0.18)		

PHA, Phytohemagglutinin-stimulated; U, unstimulated.

n can differ if single subjects had no measurements available.

*The respective risk allele was defined as the allele increasing the risk for the development of asthma as shown in previous publications.^{E1}

†P value of GMR by regression models using an allele-counting model (0, 1, 2) for coding of the respective SNP.

TABLE E4. Children with risk haplotype of 17q21 SNPs had increased IL-17 secretion in unstimulated and phytohemagglutinin-stimulated cord blood (N = 40)

Cytokine	Genotype	n	Geometric mean (95% CI)	GMR (95% CI) P value†	
				Crude	Adjusted for maternal atopy
IL-17 U	Risk haplotype*	17	0.19 (0.07-0.56)	3.04 (0.83-11.1) P = .10	3.14 (0.84-11.7) P = .10
	Nonrisk haplotype	23	0.06 (0.03-0.16)		
IL-17 PHA	Risk haplotype*	15	0.29 (0.07-1.15)	3.83 (0.84-17.4) P = .09	4.10 (0.91-18.6) P = .08
	Nonrisk haplotype	23	0.08 (0.03-0.20)		

PHA, Phytohemagglutinin-stimulated; U, unstimulated.

n can differ if single subjects had no measurements available.

**Risk haplotype* was defined as the haplotype containing all risk alleles only of the 4 tagging SNPs; *nonrisk haplotype* was defined as the haplotype containing all nonrisk alleles only of the 4 tagging SNPs. The respective risk alleles were defined as the alleles increasing the risk for the development of asthma as shown in the original publication.^{E1}

†P value of GMR by regression models (risk type compared with the nonrisk type).

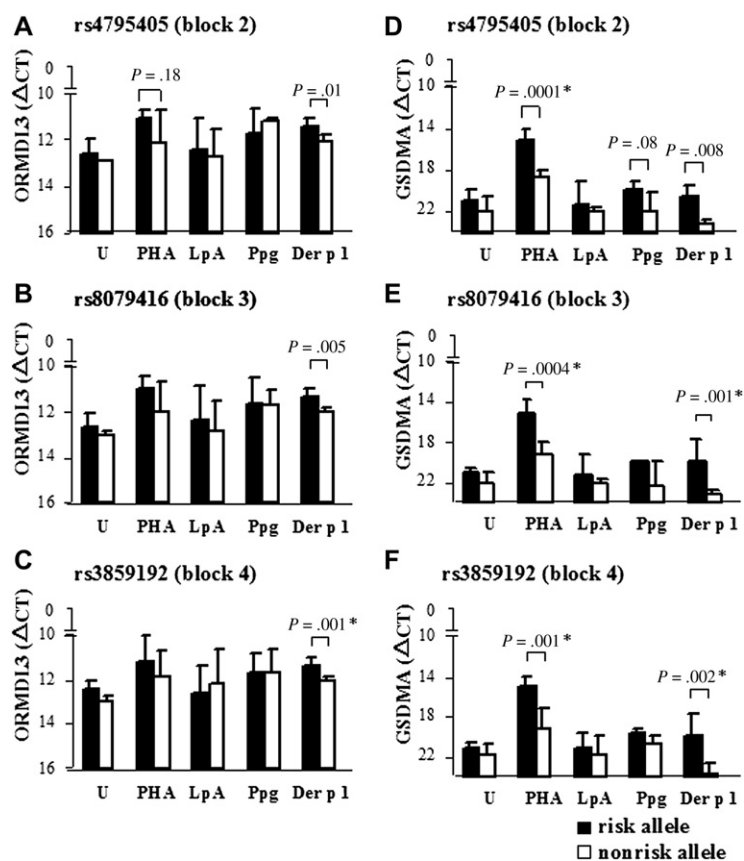


FIG E1. *ORMDL3* and *GSDMA* gene expression was increased in carriers of 17q21 risk alleles (black bars) in comparison with nonrisk alleles (white bars) after Der p 1 stimulation in CBMCs. **A** and **D**, rs4795405, $n = 12/11$. **B** and **E**, rs8079416, $n = 10/10$. **C** and **F**, rs3859192, $n = 9/10$. Δ CT values are shown; axes are vice versa; lower Δ CT values between groups correspond to increased mRNA expression. Data are shown as median ($\pm$ interquartile range). P values are calculated by the Wilcoxon test. Gene expression was assessed in subjects homozygous for the risk or nonrisk alleles. PHA, Phytohemagglutinin; Ppg, peptidoglycan; U, unstimulated. *Significance of $P \leq .004$ (below the multiple testing significance threshold).