Polymorphisms related to *ORMDL3* are associated with asthma susceptibility, alterations in transcriptional regulation of *ORMDL3*, and changes in T_H2 cytokine levels

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Background: Chromosome 17q21, harboring the orosomucoid 1-like 3 *(ORMDL3)* gene, has been consistently associated with childhood asthma in genome-wide association studies.
Objective: We investigated genetic variants in and around *ORMDL3* that can change the function of *ORMDL3* and thus contribute to asthma susceptibility.
Methods: We performed haplotype analyses and fine mapping of the *ORMDL3* locus in a cross-sectional (International Study of Asthma and Allergies in Childhood Phase II, n = 3557 total subjects, n = 281 asthmatic patients) and case-control (Multicenter Asthma Genetics in Childhood Study/International Study of Asthma and Allergies in Childhood Phase II, n = 1446 total subjects, n = 763 asthmatic patients) data set to identify putative causal single nucleotide polymorphisms (SNPs) in the locus. Top asthma-associated polymorphisms were analyzed for allele-specific effects on transcription factor binding and promoter activity *in vitro* and gene expression in PBMCs after stimulation *ex vivo*.
Results: Two haplotypes (H1 and H2) were significantly associated with asthma in the cross-sectional ($P = 9.9 \times 10^{-5}$ and $P = .0035$, respectively) and case-control ($P = 3.15 \times 10^{-8}$ and $P = .0021$, respectively) populations. Polymorphisms rs8076131 and rs4065275 were identified to drive these effects. For rs4065275, a quantitative difference in transcription factor binding was found, whereas for rs8076131, changes in upstream stimulatory factor 1 and 2 transcription factor binding were observed *in vitro* by using different cell lines and PBMCs. This might contribute to detected alterations in luciferase activity paralleled with changes in *ORMDL3* gene expression and IL-4 and IL-13 cytokine levels *ex vivo* in response to innate and adaptive stimuli in an allele-specific manner. Both SNPs were in strong linkage disequilibrium with asthma-associated 17q21 SNPs previously related to altered *ORMDL3* gene expression.
Conclusion: Polymorphisms in a putative promoter region of *ORMDL3*, which are associated with childhood asthma, alter transcriptional regulation of *ORMDL3*, correlate with changes in T_H2 cytokines levels, and therefore might contribute to the childhood asthma susceptibility signal from 17q21. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Asthma, association study, polymorphism, ORMDL3, chromosome 17q21, promoter activity, T_H2 cytokine, upstream stimulatory factor, transcriptional regulation*

A major genetic locus specific for childhood asthma on chromosome 17q21 was identified by using a genome-wide association study (GWAS)[1] and confirmed in many subsequent studies.[2-12] However, this susceptibility locus harbors many genes (IIKAROS family zinc finger 3, alias ZNFN1A3 *[IKZF3]*; zona pellucida binding protein 2 *[ZPBP2]*; gasdermin B, alias GSDML *[GSDMB]*; orosomucoid 1-like 3 *[ORMDL3]*; and gasdermin A, alias GSDM1 *[GSDMA]*) with intense and extensive linkage disequilibrium (LD) among the respective alleles. Hence these putative candidate genes might contribute to the association signal with asthma.

Although asthma-associated polymorphisms in 17q21 were first linked to orosomucoid 1-like 3 *(ORMDL3)* gene expression,[1]

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Abbreviations used

Ct:	Cycle threshold
EMSA:	Electrophoretic mobility shift assay
EXACT:	Expression Analysis Cohort
GWAS:	Genome-wide association study
ISAAC II:	International Study of Asthma and Allergies in Childhood Phase II
LD:	Linkage disequilibrium
LpA:	Lipid A
MAGICS:	Multicenter Asthma Genetics in Childhood Study
OR:	Odds ratio
ORMDL3:	Orosomucoid 1-like 3
PMA:	Phorbol 12-myristate 13-acetate
Ppg:	Peptidoglycan
SNP:	Single nucleotide polymorphism
SP:	Specificity protein
SPT:	Skin prick test
USF:	Upstream stimulatory factor
UTR:	Untranslated region

allele-specific effects of these polymorphisms on the expression of other genes in the region were subsequently also shown in adults (*GSDMB*,[12] *ORMDL3*, and *ZPBP2*[2,12]) and cord blood (*ORMDL3* and *GSDMA*[13]). Although other genes within 17q21 might contribute to the asthma association signal, *ORMDL3* remains a major asthma candidate gene in this locus. Recently, it was shown that ORMDL3 is involved in endoplasmic reticulum–mediated Ca^{2+} homeostasis[14,15] and unfolded protein response,[14,16] which potentially activate inflammatory processes[17,18] and T-lymphocyte induction.[15,19,20] The *in vitro* overexpression of *ORMDL3* in a human epithelial cell line led to the induction of metalloproteases, chemokines, and oligoadenylate synthetase genes, which are known to play a role in allergic inflammation, remodeling, and antiviral responses.[15] In mice *ORMDL3* levels in bronchial epithelial cells were inducible after allergen challenge, depending on the presence of signal transducer and activator of transcription 6.[21] Furthermore, regulation of eosinophil trafficking in a mouse model of allergic inflammation was in part attributable to *ORMLD3*.[22] A regulatory function in the metabolism of sphingolipids in *Saccharomyces cerevisiae* (ORM)[23] and in mice (ORMDL3)[24] has been demonstrated, connecting *ORMDL3*, asthma development, and the sphingolipid pathway. However, a direct link to asthma pathogenesis is still undefined.

In this study we investigated the asthma association signal from the 17q21 locus[1] in detail using a haplotype approach in combination with 1000 Genomes Project data[25,26] to overcome the strong LD in 17q21 by identifying asthma-associated genetic variants in *ORMDL3* with putative functional relevance. Subsequently, we performed *in vitro* and *ex vivo* analyses to investigate the functional effect of these variants on the *ORMDL3* gene.

METHODS

Further details on the methods used in this study are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Study populations and genetic analyses

An unbiased cross-sectional haplotype analysis to discover tagging blocks containing functionally relevant *ORMDL3* polymorphisms was performed in children from 3 German centers (total n = 4264; Munich, n = 1159; Dresden, n = 1940 for the International Study of Asthma and Allergies in Childhood Phase II [ISAAC II][27]; and Leipzig, n = 1165[28]; see Fig E1 in this article's Online Repository at www.jacionline.org). Samples that contributed to the initial GWAS discovery of the 17q21 locus[1] were excluded from this analysis, resulting in a study population of 3557 (see Table E1 in this article's Online Repository at www.jacionline.org).

Independent confirmation of these results was obtained in an asthma-enriched case-reference analysis (n = 1446), which included 691 asthmatic cases from the Multicenter Asthma Genetics in Childhood Study (MAGICS)[1,29] and 72 asthmatic cases and 683 healthy control subjects from ISAAC II (see Fig E1 and Table E2 in this article's Online Repository at www.jacionline.org).[1,27] Further details on study populations are provided in the Methods section in this article's Online Repository.

Genotyping was performed by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Sequenom, San Diego, Calif).[30] Deviation from Hardy-Weinberg equilibrium was analyzed by using χ^2 tests (see Tables E3 and E4 in this article's Online Repository at www.jacionline.org). Associations of binary traits were evaluated by using logistic regression. Odds ratios (ORs), 95% CIs, and *P* values are reported. The Benjamini-Hochberg method[31] was applied to correct for multiple testing by using the false discovery rate of an underlying significance test ($P \leq .05$). With a *P* value of .05 or less, a proportion of discoveries are expected to be false with less than 5%. Based on LD, *ORMDL3* tagging single nucleotide polymorphisms (SNPs; minor allele frequency, ≥0.03; $r^2 > 0.85$; chromosome 17, 38,072,294-38,088,854 bp) were obtained by using Haploview 4.2 (HapMap release 28)[32] and the 1000 Genomes Project (Fig 1).[25,26] *ORMDL3* single and tagging SNPs of each bin were investigated for associations with asthma and asthma subphenotypes (atopic and nonatopic asthma). The putative functional relevance of *ORMDL3* SNPs was determined by using F-SNP software (http://compbio.cs.queensu.ca/F-SNP/). Haplotype frequencies for *ORMDL3* tagging SNPs were estimated by using the expectation-maximization algorithm.[33] Global tests in Cocaphase[34] were performed (haplo.stats R package)[35] to evaluate haplotype associations. Haplotype trend regressions[36] were calculated to specify effects of individual haplotypes. If not further specified, analyses were performed with R software (version 3.0.0).[37]

Functional analyses of asthma-associated *ORMDL3* polymorphisms

To study *ORMDL3* allele–dependent promoter activity *in vitro*, HEK293 cells were transfected with the *Firefly luciferase* reporter plasmid pGL3 under the control of a putative *ORMDL3* promoter region (1069 bp) containing either both asthma risk or nonrisk alleles of rs8076131 and rs4065275, a combination of risk and nonrisk alleles, or the nonrisk allele of each SNP. The pRL-TK *Renilla luciferase* reporter plasmid (Promega, Madison, Wis) was cotransfected for normalization of transfection efficiency and cell viability. Cells were left in medium or stimulated (0.1 μmol/L ionomycin, 1 μmol/L ionomycin, or 50 ng/mL phorbol 12-myristate 13-acetate [PMA]/1 μmol/L ionomycin) 3 hours after transfection, and dual luciferase reporter assays were performed 21 hours later.

Allele-specific alterations in transcription factor binding to rs8076131 and rs4065275 were investigated by using electrophoretic mobility shift assay (EMSA) with nuclear extract from different cell lines (Jurkat T, YT, and HEK293 cells) and PBMCs before and after stimulation with PMA/ionomycin or Der p 1. Supershift and competition experiments were performed by adding either the transcription factor–specific antibody (4 μg) or unlabeled probes (as indicated: 100-, 50- or 25-fold molar excess) before adding the labeled probe. Sequences of respective probes are depicted in Table E5 in this article's Online Repository at www.jacionline.org.

ORMDL3 gene expression *ex vivo* was analyzed by using RT-PCR in PBMCs from nonasthmatic adult subjects (n = 47) recruited in the Expression Analysis Cohort (EXACT; n = 113 subjects), depending on genotype status of rs8076131 and rs4065275. PBMCs were unstimulated or stimulated for 48 hours (*Dermatophagoides pteronyssinus* [Der p 1, 30 μg/μL], lipid A [LpA; 0.1 μg/μL], peptidoglycan [Ppg; 10 μg/μL], and PHA [5 μg/μL]). Genotype-specific effects of rs8076131 and rs4065275 on cytokine secretion

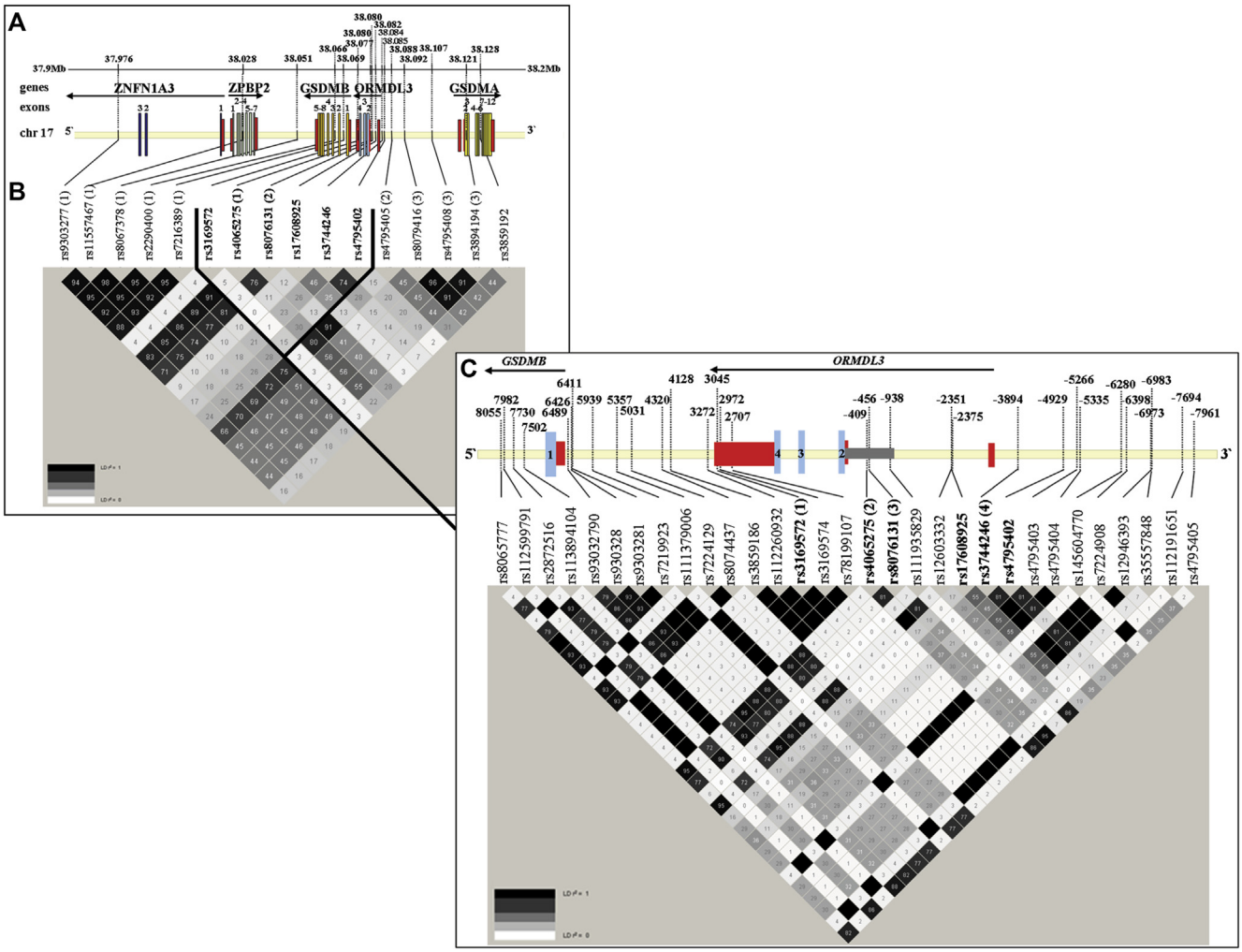

FIG 1. Genomic structure, localization, and LD (r^2 plot) of the genotyped polymorphisms within the 17q21 locus and *ORMDL3*. **A,** Genomic structure and localization of the genotyped polymorphisms within the 17q21 locus and *ORMDL3* tagging SNPs are depicted. *Arrows* indicate transcriptional direction. Exons with exon numbers are displayed in color codes. *Smaller red boxes* represent 5′ and 3′ UTRs. **B,** LD (r^2 plot) corresponding to the respective tagging bins of 17q21 and *ORMDL3* polymorphisms are shown. Tagging SNPs within *ORMDL3* included in the haplotype analyses ($r^2 > 0.85$) are depicted in boldface letters. **C,** In the *upper panel* the genomic structure and localization of the *ORMDL3* polymorphisms (relative to the translation start site) retrieved from the HapMap and the 1000 Genomes Project database are shown. *Arrows* indicate transcriptional direction of *ORMDL3*. Exons are displayed in *blue*, and *smaller red boxes* represent the 5′ and 3′ UTRs. The *ORMDL3* promoter region (1069 bp) cloned into the pGL3 basic vector for functional analyses is depicted in the *gray box* directly upstream of the translation start site. In the *lower panel* LD ($r^2 > 0.85$) corresponding to the respective tagging bins of *ORMDL3* SNPs are shown. *ORMDL3* tagging SNPs are depicted in boldface letters.

were investigated in PBMC supernatants (n = 54). For that purpose, IL-4, IL-8, and IL-13 cytokine levels were measured in duplicates (50 μL) in a Bio-Plex panel, according to the manufacturer's protocol (Bio-Rad, Munich, Germany). A 9-point standard curve was applied, and the respective detection ranges for IL-4, IL-8, and IL-13 are displayed in Table E6 in this article's Online Repository at www.jacionline.org.

RESULTS

SNPs in the 17q21 gene region are associated with asthma phenotypes in a cross-sectional population

First, we genotyped the top 10 asthma-associated SNPs (Fig 1, *A* and *B*, and see Table E3, normal font letters, in this article's Online Repository at www.jacionline.org), which also influenced *ORMDL3* expression in our initial GWAS[1] in the cross-sectional study population of 3557 children from 3 different centers (see Table E1), and confirmed their role in asthma (Table I). ORs of between 1.21 and 1.54 were observed, and the effect of 17q21 SNPs was stronger for atopic asthma (OR, 1.43-1.81) than for nonatopic asthma (OR, 1.10-1.42) compared with that seen in nonatopic nonasthmatic subjects (further details are provided in the Methods section in this article's Online Repository). Effects were found when the population was analyzed individually per center and in the overall analysis (n = 4264, data not shown).

TABLE I. Polymorphisms in the 17q21 locus are associated with asthma phenotypes in a cross-sectional study population

	Asthma (n = 281)		Atopic asthma (n = 124)		Nonatopic asthma (n = 139)	
SNP	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
rs9303277	1.42 (1.18-1.70)	.0002*	1.59 (1.21-2.08)	.0008*	1.33 (1.03-1.70)	.0280
rs11557467	1.44 (1.20-1.73)	8.23×10^{-5}*	1.62 (1.23-2.13)	.0006*	1.36 (1.06-1.76)	.0165
rs8067378	1.44 (1.20-1.73)	7.71×10^{-5}*	1.64 (1.25-2.16)	.0004*	1.35 (1.05-1.73)	.0197
rs2290400	1.47 (1.23-1.77)	3.47×10^{-5}*	1.70 (1.29-2.25)	.0002*	1.38 (1.07-1.78)	.0139
rs7216389	1.48 (1.23-1.77)	3.09×10^{-5}*	1.70 (1.29-2.24)	.0002*	1.39 (1.07-1.79)	.0120
rs4795405	1.54 (1.28-1.86)	6.25×10^{-6}*	1.81 (1.36-2.40)	4.45×10^{-5}*	1.42 (1.09-1.84)	.0083
rs8079416	1.30 (1.09-1.55)	.0038	1.54 (1.18-2.01)	.0013*	1.18 (0.92-1.51)	.1856
rs4795408	1.26 (1.06-1.51)	.0103	1.49 (1.14-1.95)	.0036	1.16 (0.91-1.49)	.2421
rs3894194	1.26 (1.05-1.50)	.0121	1.47 (1.12-1.91)	.0048	1.18 (0.92-1.51)	.1911
rs3859192	1.21 (1.01-1.44)	.0499	1.43 (1.10-1.87)	.0079	1.10 (0.86-1.42)	.4437

ORs (95% CIs) for associations of 17q21 SNPs with asthma, atopic asthma, and nonatopic asthma in the cross-sectional study population (ISAAC II, n = 3557) are displayed in the order of their chromosomal location.
*Significant after correction for multiple testing.

TABLE II. Haplotype analyses for *ORMDL3* tagging SNPs in the cross-sectional study population (ISAAC II, n = 3557)

	Tagging SNPs of the *ORMDL3* locus*				
		Estimated frequencies		HTR	
No. of cases, global *P* value	Common haplotypes†	Cases (%)	Control subjects (%)	OR (95% CI)	*P* value (HTR)
Asthma (n = 259), *P* = .0022					
H1	G-G-A-T-C-C	58.39	49.47	2.08 (1.43-3.01)	.0001
H2	G-A-G-T-C-C	19.69	25.40	0.51 (0.32-0.80)	.0037
H3	G-A-G-C-T-A	8.30	9.47	0.75 (0.39-1.43)	.3773
H4	G-A-G-T-T-A	6.37	8.94	0.47 (0.23-0.99)	.0455
H5	A-A-A-T-C-A	4.93	4.37	1.33 (0.59-3.03)	.4952
All rare		2.32	2.35		
Atopic asthma (n = 113), *P* = .0010					
H1	G-G-A-T-C-C	60.92	48.99	2.63 (1.51-4.59)	.0006
H2	G-A-G-T-C-C	15.49	25.63	0.27 (0.13-0.58)	.0006
H3	G-A-G-C-T-A	9.73	9.16	1.14 (0.46-2.83)	.7757
H4	G-A-G-T-T-A	5.30	9.45	0.28 (0.09-0.93)	.0377
H5	A-A-A-T-C-A	5.89	4.41	2.04 (0.67-6.24)	.2114
All rare		2.67	2.36		
Nonatopic asthma (n = 128), *P* = .2655					
H1	G-G-A-T-C-C	56.76	48.99	1.88 (1.12-3.16)	.0164
H2	G-A-G-T-C-C	22.92	25.63	0.75 (0.41-1.37)	.3483
H3	G-A-G-C-T-A	7.03	9.16	0.56 (0.21-1.49)	.2444
H4	G-A-G-T-T-A	7.03	9.45	0.52 (0.19-1.39)	.1912
H5	A-A-A-T-C-A	4.30	4.41	0.95 (0.27-3.26)	.9292
All rare		1.96	2.36		

HTR, Haplotype trend regression.
*rs3169572, rs4065275, rs8076131, rs17608925, rs3744246, and rs4795402.
†Common haplotypes with a haplotype frequency of 3% or greater.

SNPs in the proximal *ORMDL3* promoter region drive the association between asthma and *ORMDL3* haplotypes in a cross-sectional and a case-control population

Next, we fine mapped the *ORMDL3* gene region spanning from 5 kb upstream to 5 kb downstream of the gene. Using available genotyping information from the HapMap database and the 1000 Genomes data set, we identified 31 SNPs with a minor allele frequency of 0.03 (Fig 1, *C*, and see Table E7 in this article's Online Repository at www.jacionline.org): in total, 27 SNPs clustered in 4 tagging bins, and an additional 4 single SNPs were identified. All SNPs were analyzed *in silico* for their putative influence on *ORMDL3* gene function by using F-SNP software. For all further analyses, only single SNPs or SNP tagging bins containing polymorphisms with a potential functional effect on *ORMDL3* regulation were included (see Table E8 in this article's Online Repository at www.jacionline.org).

Accordingly, 6 haplotype tagging SNPs were identified (Fig 1, *C*). Haplotype analyses were performed in 3287 children of the cross-sectional study population for whom complete phased haplotype information was available, with 5 haplotypes reaching a frequency of 3% or greater (Tables II and III). Haplotypes H1 and H2 showed significant associations with asthma and an even stronger effect size with atopic asthma (Table II) versus that seen in nonatopic nonasthmatic subjects. Interestingly, association signals seem to be mainly driven by rs4065275 and

TABLE III. Haplotype analyses for *ORMDL3* tagging SNPs in the case-control study population (MAGICS/ISAAC II, n = 1446)

No. of cases, global *P* value	Tagging SNPs of the *ORMDL3* locus*			HTR	
		Estimated frequencies			
	Common haplotypes†	Cases (%)	Control subjects (%)	OR (95% CI)	*P* value (HTR)
Asthma (n = 763), $P = 9.14 \times 10^{-7}$					
H1	G-G-A-T-C-C	56.68	46.09	2.30 (1.71-3.10)	3.15×10^{-8}
H2	G-A-G-T-C-C	21.65	26.51	0.58 (0.41-0.82)	.0021
H3	G-A-G-C-T-A	7.67	11.11	0.44 (0.26-0.75)	.0023
H4	G-A-G-T-T-A	8.48	8.58	0.93 (0.55-1.59)	.7954
H5	A-A-A-T-C-A	3.73	4.64	0.63 (0.30-1.30)	.2107
All rare		1.79	3.07		
Atopic asthma (n = 548), $P = .0142$					
H1	G-G-A-T-C-C	55.60	48.04	1.81 (1.26-2.59)	.0013
H2	G-A-G-T-C-C	22.27	25.09	0.72 (0.47-1.11)	.1352
H3	G-A-G-C-T-A	7.83	9.70	0.62 (0.32-1.18)	.1449
H4	G-A-G-T-T-A	8.65	8.87	0.94 (0.49-1.81)	.8461
H5	A-A-A-T-C-A	3.83	4.73	0.64 (0.27-1.53)	.3138
All rare		1.82	3.57		
Nonatopic asthma (n = 113), $P = .1430$					
H1	G-G-A-T-C-C	57.86	48.04	2.09 (1.18-3.72)	.0121
H2	G-A-G-T-C-C	17.78	25.09	0.41 (0.19-0.87)	.0208
H3	G-A-G-C-T-A	7.63	9.70	0.59 (0.19-1.82)	.3540
H4	G-A-G-T-T-A	9.54	8.87	1.17 (0.41-3.36)	.7692
H5	A-A-A-T-C-A	4.42	4.73	0.85 (0.22-3.40)	.8232
All rare		2.77	3.57		

HTR, Haplotype trend regression.
*rs3169572, rs4065275, rs8076131, rs17608925, rs3744246, and rs4795402.
†Common haplotypes with a haplotype frequency of 3% or greater.

TABLE IV. Association analyses for *ORMDL3* tagging SNPs in the cross-sectional study population (ISAAC II, n = 3557)

SNP*	Asthma (n = 281)		Atopic asthma (n = 124)		Nonatopic asthma (n = 139)	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Rs3169572	1.16 (0.78-1.74)	.4671	1.37 (0.79-2.39)	.2663	1.02 (0.56-1.85)	.9457
Rs4065275	1.47 (1.23-1.77)	3.04×10^{-5}†	1.69 (1.28-2.22)	.0002†	1.38 (1.07-1.78)	.0137
rs8076131	1.52 (1.26-1.83)	9.45×10^{-6}†	1.85 (1.39-2.46)	2.10×10^{-5}†	1.38 (1.07-1.79)	.0140
rs17608925	1.13 (0.83-1.53)	.4388	1.08 (0.7-1.65)	.7342	1.28 (0.81-2.04)	.2886
rs3744246	1.34 (1.05-1.72)	.0198	1.27 (0.88-1.82)	.1965	1.47 (1.03-2.1)	.0346
rs4795402	1.20 (0.97-1.49)	.1011	1.1 (0.8-1.5)	.5660	1.33 (0.97-1.81)	.0755

ORs (95% CIs) for associations of *ORMDL3* tagging SNPs with asthma, atopic asthma, and nonatopic asthma in the cross-sectional study population (ISAAC II, n = 3557) are displayed in order of their respective chromosomal location.
*Identified *ORMDL3* tagging SNPs based on an r^2 value of greater than 0.85.
†Significant after correction for multiple testing.

rs8076131 because H1 (G-G-A-T-C-C) contains the respective asthma risk alleles, whereas H2 (G-A-G-T-C-C) comprises the corresponding nonrisk alleles. Similar effects were observed for H1 and H2 in the case-control population (Table III). In addition, a significant association signal with asthma was detectable for H3 (G-A-G-C-T-A).

The 2 SNPs (rs8076131 and rs4065275) driving the association are located approximately 500 bp upstream of the coding region in a putative *ORMDL3* promoter region. These SNPs were in strong LD with polymorphisms previously found to be associated with asthma and *ORMDL3* expression (Fig 1, *A* and *B*, and see Table E3, bold letters).[1] Additionally, among the genotyped *ORMDL3* polymorphisms, rs8076131 and rs4065275 showed the strongest effect on asthma susceptibility in the cross-sectional (Table IV, and see Table E4) and case-control populations (Table V). Therefore both SNPs were analyzed further as candidates for functional analyses.

ORMDL3 SNPs rs8076131 and rs4065275 change transcription factor binding and influence the activity of an *ORMDL3* promoter region in an allele-specific manner

The allele-specific effect of rs8076131 and rs4065275 on *ORMDL3* promoter activity was tested in a luciferase reporter system in HEK293 cells by using pGL3 plasmids containing different combinations of the 2 alleles in the putative *ORMDL3* promoter region (Fig 2, *A*). Strongly significant differences in luciferase activity were observed for all 4 *ORMDL3* constructs before and after stimulation with PMA/ionomycin. In the presence of both asthma risk alleles of rs8076131 and rs4065275, higher levels of *in vitro* luciferase activity were detectable compared with those for the *ORMDL3* construct carrying the respective nonrisk alleles in stimulated cells. Both SNPs seemed to contribute to the promoter activity because a significant decrease in luciferase activity was observed if only one *ORMDL3*

TABLE V. Association analyses for *ORMDL3* tagging SNPs in the case-control study population (MAGICS/ISAAC II, n = 1446)

	Asthma (n = 763)		Atopic asthma (n = 548)		Nonatopic asthma (n = 113)	
SNP*	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
rs3169572	1.43 (0.99-2.06)	.0549	1.40 (0.91-2.17)	.1283	1.30 (2.63-0.64)	.4656
rs4065275	1.49 (1.28-1.73)	3.36×10^{-7}†	1.34 (1.11-1.61)	.0024	1.51 (1.12-2.04)	.0070
rs8076131	1.41 (1.21-1.64)	7.78×10^{-6}†	1.26 (1.05-1.51)	.0148	1.37 (1.01-1.85)	.0429
rs17608925	1.47 (1.13-1.90)	.0041	1.23 (0.88-1.71)	.2273	1.17 (0.68-2.00)	.5662
rs3744246	1.24 (1.03-1.50)	.0239	1.15 (0.91-1.45)	.2408	1.06 (0.73-1.54)	.7499
rs4795402	1.30 (1.09-1.55)	.0032	1.21 (0.97-1.50)	.0899	1.15 (0.81-1.63)	.4418

ORs (95% CIs) for associations of *ORMDL3* tagging SNPs with asthma, atopic asthma, and nonatopic asthma in the case-control data set (n = 1446) are displayed in the order of their respective chromosomal location.
*Identified *ORMDL3* tagging SNPs based on an r^2 value of greater than 0.85.
†Significant after correction for multiple testing.

risk allele was present in the respective constructs. Of note, when this *ORMDL3* promoter region was cloned 3′5′ upstream of the luciferase gene, no luciferase activity was detectable. These results confirmed that this region indeed acts as a promoter region rather than an intronic enhancer (data not shown).

EMSAs were performed to analyze whether *in silico* predicted allele-specific changes of transcription factor binding at both loci are present, which could contribute to the observed effects on luciferase activity. Indeed, the nonrisk G allele at rs8076131 leads to a novel DNA/protein complex (Fig 2, *B*, lanes 12 and 13) containing upstream stimulatory factor (USF) 1 and USF2 transcription factors in Jurkat T cells before and after stimulation with PMA/ionomycin (lanes 16, 18, and 19) and Der p 1 (see Fig E3, lanes 3 and 5, in this article's Online Repository at www.jacionline.org) and PBMCs before and after stimulation (PMA/ionomycin, or Der p 1; Fig 3, *A*, lanes 4, 9, and 10), which was not detectable in the presence of the risk A allele (Fig 2, *B*, lanes 2 and 3, and Fig 3, lanes 1-3). Similar effects after stimulation with PMA/ionomycin were observed by using nuclear extracts of YT and HEK293 cells (see Fig E2 in this article's Online Repository at www.jacionline.org).

At the site harboring rs4065275, binding of the transcription factors specificity protein (SP) 1, SP3, SP4, USF1, and USF2 was demonstrated by using specific consensus sites and antibodies (Fig 2, *C*, lanes 4-10; Fig 3, *B*, lanes 4-8; and see Fig E4, *B*, lanes 5-9, in this article's Online Repository at www.jacionline.org), with no qualitative difference for the 2 allelic variants. However, quantitative binding affinity of these transcription factors seemed to be allele specific in nuclear extract from Jurkat T cells before (see Fig E4, *A*, lanes 1 vs 6) and after stimulation with PMA/ionomycin (Fig 2, *C*, lanes 1 vs 11) and Der p 1 (see Fig E4, *A*, lanes 1 vs 10) in YT, HEK293 (data not shown), and primary cells (PBMCs; Fig 3, *B*, lanes 3 vs 9). In the presence of the risk G allele for rs4065275, the addition of a 100-fold molar excess of unlabeled rs4065275_A oligonucleotide (Fig 2, *C*, lane 3) and the consensus sites for SPs (Fig 2, *C*, lane 4) and USF (Fig 2, *C*, lane 5) disrupted the respective complex formation, whereas similar competition experiments for the probe carrying the nonrisk A allele retained some of the binding ability (Fig 2, *C*, lanes 13 and 15). To confirm these findings, additional cross-competition experiments of cold rs4065275 probes either carrying the risk G or the nonrisk A allele were performed. By using nuclear extract from unstimulated or Der p 1–stimulated Jurkat T cells, cross-competition with decreasing fold molar excess (×100, ×50, and ×25) of unlabeled oligos confirmed that rs4065275_G could not fully repress the SP complexes emerging with incubation of rs4065275_A (see Fig E4, *A*, lanes 8-10, and see Fig E4, *B*, lanes 12-14). In contrast, the oligo containing the nonrisk A allele of rs4065275 was able to entirely abrogate the signal of rs4065275_G, even using only 25-fold molar excess of unlabeled oligos (see Fig E4, *A*, lanes 3-5, and see Fig E4, *B*, lanes 3-4). Hence it was concluded that transcription factors of the SP family indeed revealed a stronger binding affinity in the presence of nonrisk A allele of rs4065275.

Polymorphisms rs8076131 and rs4065275 change gene expression of *ORMDL3* and IL-4 and IL-13 cytokine levels in PBMCs after stimulation *ex vivo*

Allele-specific *ORMDL3* mRNA expression was measured in PBMCs (n = 47) before and after exposure to innate and adaptive stimuli to study the role of rs8076131 and rs4065275 on transcriptional activity of *ORMDL3*. Overall, *ORMDL3* expression was significantly inducible after stimulation with Ppg ($P = .006$) and PHA ($P = 1.45 \times 10^{-9}$; Fig 4, *A*). The genotype status of each subject was determined to further study the effect of the functionally relevant *ORMDL3* promoter polymorphisms rs8076131 and rs4065275 on *ex vivo ORMDL3* levels. No significant genotype-dependent differences in *ORMDL3* gene expression were detectable at baseline. Subjects homozygous for both risk alleles (Fig 4, *B*, black bar) versus nonrisk alleles (Fig 4, *B*, white bar) for the SNPs rs8076131 and rs4065275 revealed significantly increased *ORMDL3* levels after stimulation with Der p 1 ($P = .050$). A stronger effect was observed for rs8076131 alone (Fig 3, *D*) because *ORMDL3* gene expression was significantly altered after stimulation with Der p 1 ($P = .007$), LpA ($P = .013$), Ppg ($P = .043$), and PHA ($P = .022$). A similar but not significant trend was detectable for rs4065275 (Fig 4, *C*).

In the same samples IL-4 and IL-13 cytokine levels dependent on these *ORMDL3* promoter polymorphisms were measured in the supernatants of cultured PBMCs (n = 54) before and after stimulation. Hence median levels of IL-4 (Der p 1, $P = .044$; PHA, $P = .076$; Fig 5, *A*) and IL-13 (PHA, $P = .006$; Fig 5, *B*) were significantly increased in subjects homozygous for the risk allele of rs8076131. Correspondingly, in the presence of the risk alleles of rs4065275, greater IL-4 (Der p 1, $P = .086$; Fig 5, *A*) and IL-13 (Der p 1, $P = .073$; PHA, $P = .023$; Fig 5, *B*) cytokine expression was observed. Of note, IL-8 was not detectable in these samples (data not shown). These results show that an increase in *ORMDL3* gene expression mediated by

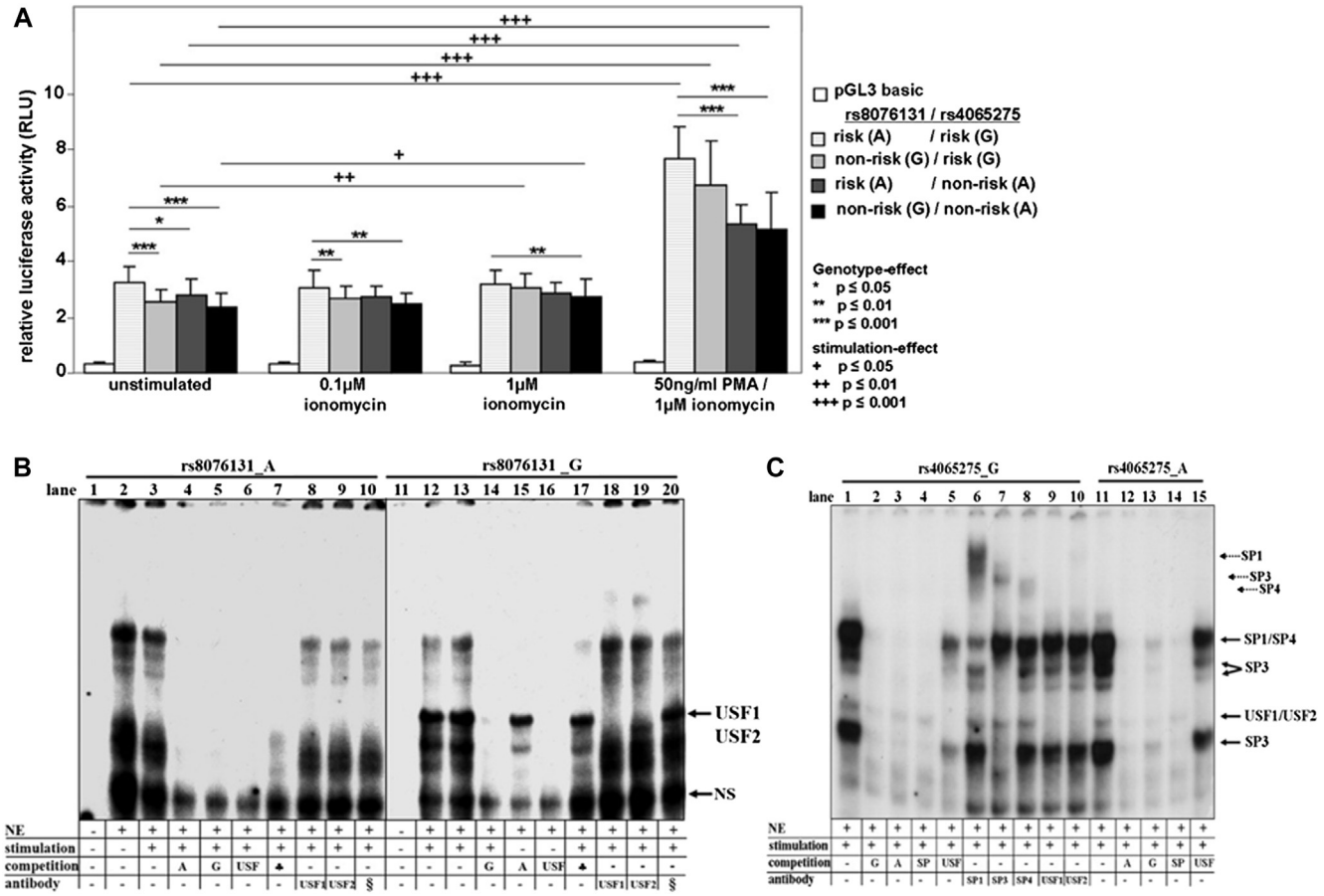

FIG 2. Asthma-associated polymorphisms in an *ORMDL3* promoter region alter *in vitro* promoter activity and transcription factor binding. **A,** HEK293 were transiently transfected with 4 different reporter constructs of an *ORMDL3* promoter region (1069 bp) carrying either both risk alleles for each of the asthma-associated SNPs (rs8076131: A, rs4065275: G), both nonrisk alleles (rs8076131: G, rs4065275: A), or a combination of the risk and nonrisk alleles (rs8076131: nonrisk G, rs4065275: risk G and rs8076131: risk A, rs4065275: nonrisk A; n = 5, triplicates). Cells were left in medium or stimulated with 0.1 μmol/L ionomycin, 1 μmol/L ionomycin, or 50 ng/mL PMA/1 μmol/L ionomycin 3 hours after transfection and harvested 24 hours after transfection. Luciferase activity was normalized for transfection efficiency by using the control plasmid pRL-TK. The relative luciferase activity is presented in relative light units *(RLU)*. **B,** EMSA analysis with an *ORMDL3* probe either carrying the risk A (rs8076131_A, *lanes 1-10*) or the nonrisk G (rs8076131_G, *lanes 1-20*) allele of rs8076131 was performed with nuclear extract *(NE)* from the Jurkat T-cell line (5 μg) cultured for 3 hours either left in medium *(−)* or stimulated *(+)* with PMA/ionomycin (50 ng/mL and 1 μmol/L, respectively). Competitors (100-fold molar excess) and supershift antibodies (4 μg) for each experiment are noted below the respective lanes. ♣, Competition experiment with an unrelated oligo; §, supershift experiment with an unrelated antibody. **C,** EMSA analysis with an *ORMDL3* probe either carrying the risk G (rs4065275_G, *lanes 1-10*) or the nonrisk A (rs4065275_A, *lanes 11-15*) allele of rs4065275 was performed with nuclear extract *(NE)* from the Jurkat T-cell line (5 μg) cultured for 3 hours either left in medium *(−)* or stimulated *(+)* with PMA/ionomycin (50 ng/mL and 1 μmol/L, respectively). Competitors (100-fold molar excess) and supershift antibodies (4 μg) for each experiment are noted below the respective lanes. Genotype-effect: $^{*}P \leq .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$; stimulation effect: $^{+}P \leq .05$, $^{++}P \leq .01$, and $^{+++}P \leq .001$.

the asthma risk alleles of rs8076131 and rs4065275 subsequently leads to induction of IL-4 and IL-13 secretion.

DISCUSSION

A major genetic susceptibility locus for childhood asthma was identified by means of GWAS[1] on chromosome 17q21 and confirmed in many subsequent studies.[2-12] However, this locus harbors numerous potentially associated genes, none of which was a candidate gene for asthma before the GWAS. Because genetic variants are tightly linked within the locus, functional approaches are needed to dissect the causal contribution of genes in the region toward asthma development. In this study we identified genetic variants in regulatory regions of *ORMDL3* associated with asthma and *ORMDL3* expression.[1] Haplotype analyses indicated that this previously identified asthma association signal seemed to be driven considerably by rs8076131 and rs4065275 in our cross-sectional and case-control populations. At the site harboring rs4065275, a quantitative difference in transcription factor binding was suggested by using EMSA. For rs8076131, we identified an allele-specific binding of USF1 and USF2 transcription factors in different cell lines. These results might explain the increase in *ORMDL3* promoter activity *in vitro* and *ORMDL3* mRNA expression and T_H2 cytokine levels *ex vivo* in the presence of asthma risk alleles.

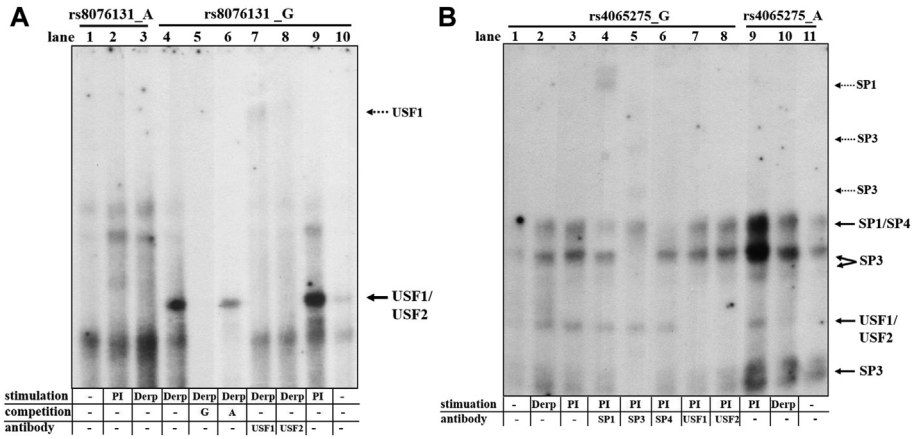

FIG 3. Asthma-associated polymorphisms in an *ORMDL3* promoter region alter transcription factor binding in PBMCs. **A,** EMSA analysis with an *ORMDL3* probe either carrying the risk A (rs8076131_A, *lanes 1-10*) or the nonrisk G (rs8076131_G, *lanes 11-20*) allele of rs8076131 was performed with nuclear extract *(NE)* from PBMCs (5 μg) cultured for 3 hours and either left in medium *(−)* or stimulated *(+)* with PMA/ionomycin (50 ng/mL and 1 μmol/L, respectively) or Der p 1 (30 μg/μL). Competitors (100-fold molar excess) and supershift antibodies (4 μg) for each experiment are noted below the respective lanes. **B,** EMSA analysis with an *ORMDL3* probe either carrying the risk G (rs4065275_G, *lanes 1-8*) or the nonrisk A (rs4065275_A, *lanes 9-11*) allele of rs4065275 was performed with nuclear extract *(NE)* from the PBMCs (5 μg) cultured for 3 hours either left in medium *(−)* or stimulated *(+)* with Der p 1 (30 μg/μL) or PMA/ionomycin (50 ng/mL and 1 μmol/L, respectively). Supershift antibodies (4 μg) for each experiment are noted below the respective lanes.

We replicated and extended the original association signal from chromosome 17q21 in the cross-sectional population and showed that atopic asthma is the primary phenotype associated with genetic variations from 17q21 but not atopic sensitization *per se*. This might indicate that effects of 17q21 SNPs on asthma susceptibility are aggravated by the presence of atopy but might not act through mechanisms of atopic sensitization itself. However, this effect was not present in the case-control population.

Although it cannot be excluded that other genes within 17q21 might contribute to the association signal, *ORMDL3* remains a leading candidate gene. The *ORMDL3* promoter is demethylated, and *ORMDL3*, in contrast to other 17q21 genes, is expressed in tissues and in resting and activated subcell populations ($CD4^+$, $CD8^+$, $CD19^+$, and mononuclear cells).[1,2,38] Similarly, we detected *ORMDL3* expression in Jurkat T, HEK293, and YT cells, although with higher levels compared with PBMCs (data not shown). Therefore one might speculate that genetic variants in regulatory regions of *ORMDL3* could lead to its dysregulation and, as a consequence and through mechanisms not yet well understood, increases disease susceptibility in the lung.

Because of intense and extensive LD across the 17q21 locus and the influence of asthma-associated 17q21 SNPs on *ORMDL3* expression, we investigated whether *ORMDL3* might harbor SNPs in regulatory regions that could be responsible for such an effect. Polymorphisms rs8076131 and rs4065275, which are strongly linked to previously studied 17q21 SNPs and located in a putative promoter region of *ORMDL3*, showed the strongest association with asthma. Attributable to the high LD with neighboring SNPs on 17q21, it would be difficult, if not impossible, to speculate on the causality of these SNPs based only on statistical data. Therefore functional analyses for rs8076131 and rs4065275 were performed. Both polymorphisms are located in intron 2 in close proximity to the translation start site in exon 2. In HEK293 cells Jin et al[39] identified multiple transcriptional start sites used by *ORMDL3* and defined a minimal promoter in the −84/+58 region relative to the transcription start site. Similar results on the transcriptional regulation of *ORMDL3* were provided by means of deletion analyses of different fragments, including 1.5 kb upstream of the 5′ untranslated region (UTR) with a 68-bp region as the functional minimal promoter in multiple cell lines.[40] We extended these findings to a region containing an alternative promoter directly upstream of the first coding exon, including rs8076131 and rs4065275, because the luciferase expression signal was dependent on its cloning orientation (data not shown).

Polymorphisms rs8076131 and rs4065275 are not entirely independent of each other, with an r^2 value of 0.7. The functional contribution of each of these asthma-associated polymorphisms was investigated to further delineate the mechanisms of *ORMDL3* transcriptional regulation depending on single SNP or haplotype effects of rs8076131 and rs4065275 *in vitro* and *ex vivo*. The strongest increase in luciferase expression, reflecting *in vitro ORMDL3* promoter activity, was detected in the presence of both asthma risk alleles for rs8076131 and rs4065275, but each SNP seemed to contribute to the signal independently at baseline and after stimulation. The relevance of these *ORMDL3* SNPs was further strengthened because *ORMDL3* gene expression was significantly increased *ex vivo* in PBMCs from patients carrying the homozygous risk haplotype only after stimulation with Der p 1. Risk alleles of rs8076131 altered *ORMDL3* levels significantly, whereas rs4065275 showed only trends in the same direction. This haplotype-specific *ORMDL3* expression is in line with recent studies in neonates, which demonstrated an allele-specific effect on *ORMDL3* expression after Der p 1 stimulation.[13] Der p 1 induces the extracellular signal-regulated kinase 1/2 signaling pathway, subsequently releasing IL-8,[41] a major chemoattractant with increased levels in the sputum[42,43] and breath condensate[44] of asthmatic patients. Overexpression of ORMDL3 in the airway epithelial cell line A549 was shown

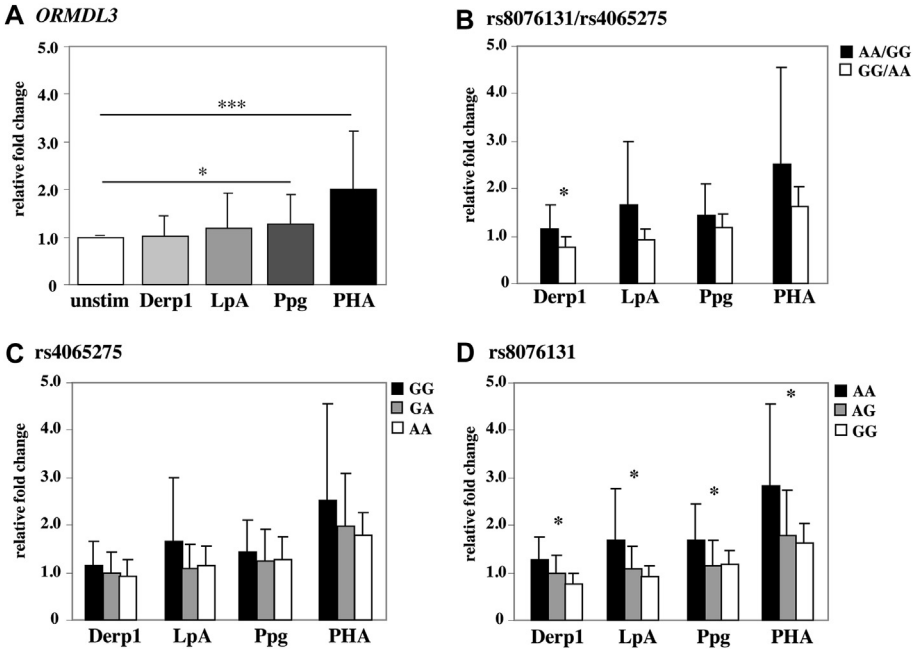

FIG 4. *ORMDL3* gene expression correlated with rs8076131 and rs4065275 in PBMCs after stimulation. **A,** *ORMDL3* mRNA levels in PBMCs (n = 47) before (*unstim*, unstimulated) and after stimulation with *Dermatophagoides pteronyssinus* (Der p 1), LpA, Ppg, and PHA. **B,** *ORMDL3* mRNA levels in PBMCs before and after stimulation depending on rs8076131 and rs4065275 (homozygous risk allele carriers AA/GG: n = 8, *black bars*; homozygous nonrisk allele carriers GG/AA: n = 8, *white bars*). **C,** *ORMDL3* mRNA levels in PBMCs before and after stimulation depending on rs4065275 (homozygous risk allele carriers GG: n = 8, *black bars*; heterozygous GA: n = 27, *gray bars*; homozygous nonrisk allele carriers AA: n = 12, *white bars*). **D,** *ORMDL3* mRNA levels in PBMCs before and after stimulation depending on rs8076131 (homozygous risk allele carriers AA: n = 12, *black bars*; heterozygous AG: n = 28, *gray bars*; homozygous nonrisk allele carriers GG: n = 8, *white bars*).Relative fold changes are shown (±SD), applying the Wilcoxon test (Fig 4, *A*) and linear regression (Fig 4, *B-D*). *$P < .05$ and ***$P < .0001$.

to increase IL-8 levels (among other chemokines).[21] In allergic asthmatic patients significantly higher *ORMDL3* levels were observed after Der p 1 stimulation, whereas in nonasthmatic control subjects *ORMDL3* was not inducible by Der p 1 (Toncheva, Potaczek, and Schedel, et al, manuscript submitted). Thus (genetically determined) ORMDL3 overexpression might be a link between allergen exposure and airway inflammation in asthmatic patients. Because IL-8 cytokine expression was less than the limit of detection in the supernatants of cultured PBMCs, we were unable to directly address this hypothesis. Hence more research on this topic needs to be undertaken to further define the mechanism behind these findings.

ORMDL3 gene transcription has thus far been demonstrated to be regulated by the transcription factors Ets-1, p300, and CREB *in vitro*.[39] In the same study patients with recurrent wheeze revealed increased levels of these transcription factors in peripheral blood in comparison with healthy control subjects. In human subjects[40] and mice,[21] *ORMDL3* activity was increased in response to IL-4 and IL-13 in a signal transducer and activator of transcription 6–dependent manner. Our data suggest a potential feedback mechanism because in the presence of the asthma risk alleles for rs8076131 and rs4065275, increased *ORMDL3* gene expression, paralleled by increased IL-4 and IL-13 levels, was detected. ORMDL3 has been shown to regulate calcium homeostasis,[14,15] which in turn mediates many aspects of T-cell responses, including T_H2 cytokine production.[19,20] These findings support a pivotal role for ORMDL3 in immune regulation.

In the current study it was shown in different cell lines and primary cells that the asthma risk allele of the SNP rs8076131 abolished the ability of the USF1/USF2 complex to bind and that the asthma risk allele of rs4065275, where USF1/USF2 also binds, seemed to change the binding affinity of the SP family (SP1, SP3, and SP4). Because similar allele-specific effects on transcription factor binding were observed in different cell lines, as well as primary cells, it is suggested that similar transcriptional regulators in different nuclear environments are likely driving *ORMDL3* promoter activity *in vitro*, as well as *ORMDL3* mRNA expression *ex vivo*. Because transcription factor binding overlaps in both loci, which are only separated by 46 bp, chromatin immune precipitation for *in vivo* analysis of transcription factor binding is technically not possible in that locus. SP and USF protein members are ubiquitously expressed with tissue- and development-specific properties.[45-47] Synergistic interactions between these transcription factors in promoter regions have been shown for a variety of genes.[48-50] Depending on the promoter context and nuclear environment of certain cell types, the concomitant control of SPs can positively or negatively affect transcriptional activity.[47] USF1 and USF2 belong to the basic/helix-loop-helix/leucine zipper family binding to Enhancer Box elements with the consensus site CANNTG.[45,46] In the presence of the rs8076131 risk allele, this Enhancer Box (CAGATG → TAGATG) site is potentially eliminated, leading to loss of USF1/USF2 binding ability. Increased *ORMDL3* expression was observed *in vitro*, as well as in primary cells in the presence

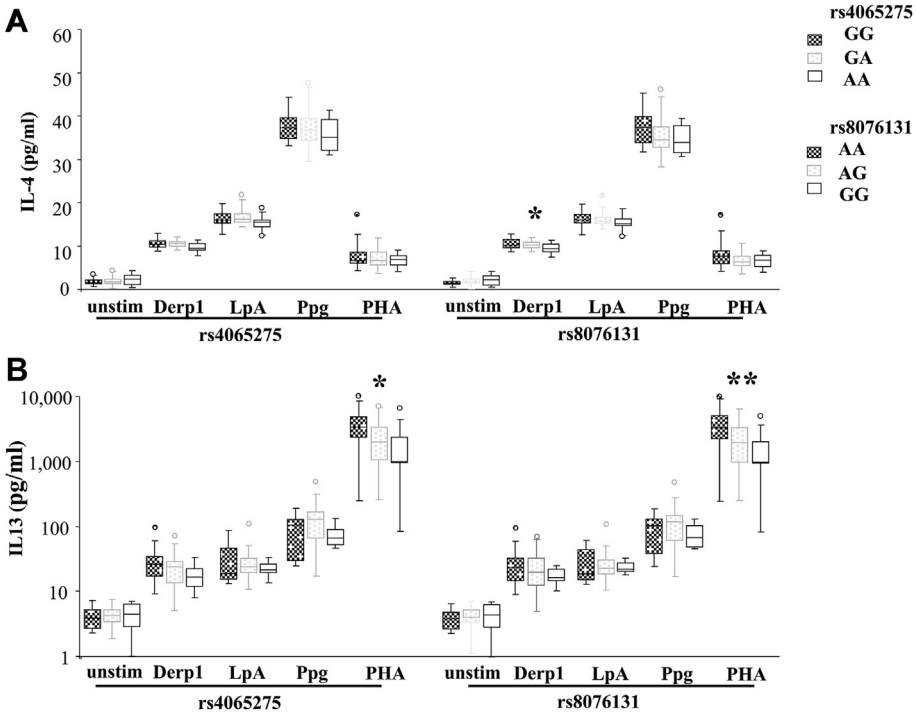

FIG 5. IL-4 and IL-13 cytokine secretion in supernatants of stimulated PBMCs is influenced by rs8076131 and rs4065275. **A,** IL-4 cytokine levels in supernatants of cultured PBMCs (n = 54) before (*unstim*, unstimulated) and after stimulation with *Dermatophagoides pteronyssinus* (Der p 1), LpA, Ppg, and PHA. **B,** IL-13 cytokine levels in supernatants of cultured PBMCs (n = 54) before (*unstim*, unstimulated) and after stimulation with *Dermatophagoides pteronyssinus* (Der p 1), LpA, Ppg, and PHA. Allele-specific effects were calculated by using a linear regression model. $*P < .05$ and $**P < .01$.

of *ORMDL3* asthma risk alleles. This result can be explained by the fact that the polymorphisms rs8076131 and rs4065275 are in very close vicinity. Conversely, it is suggested that in the presence of both nonrisk alleles, lower *ORMDL3* expression levels are observed because of enhanced binding of SPs to the nonrisk A allele of rs4065275, which subsequently converts USF1/USF2 from a transcriptional activator to a repressor. It is interesting to note that similar effects have been described for the regulation of the proximal promoter of Src-suppressed C kinase substrate[49] and the deoxycytidine kinase.[50] Furthermore, USF1 and SP1 have been reported to mediate epigenetic modifications.[51,52] In cord blood samples it was shown that an *ORMDL3* region in close vicinity to rs8076131 and rs4065275 was hypermethylated in early asthmatic patients versus nonasthmatic subjects.[53] However, whether this effect is genotype dependent and potentially related to the observed changes in transcription factor binding to the *ORMDL3* promoter regions needs to be further investigated.

Taken together, our data provide novel functional evidence that rs8076131 and rs4065275, both of which are located in the *ORMDL3* promoter region, might causally contribute to the asthma susceptibility signal from 17q21. Although further mechanistic insight into how ORMDL3 promotes asthma has yet to be provided, our data strengthen the hypothesis that *ORMDL3* contributes significantly to the asthma susceptibility signal from chromosome 17q21.

We thank Sonja Zeilinger, Ilona Dahmen, Christian Schröter, and Luise Worth for their valuable assistance and contributions to data acquisition.

Key messages

- ***ORMDL3* polymorphisms in a regulatory region of *ORMDL3* are associated with asthma in a cross-sectional and a case-control population.**
- **Asthma-associated *ORMDL3* polymorphisms alter transcription factor binding, *ORMDL3* promoter activity *in vitro*, *ORMDL3* gene expression, and T_H2 cytokine levels after stimulation with innate and adaptive stimuli.**

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METHODS

Study population

In our previous GWAS on childhood asthma, 1422 children of German or Austrian ethnicity (728 asthmatic patients) were chip genotyped by using the Illumina Sentrix HumanHap300 BeadChip (Illumina, San Diego, Calif) and had full information on asthma status available.[E1,E2] With this data set, we have also contributed to the meta-analysis by the GABRIEL Consortium.[E3] Most of the asthmatic cases (n = 655) were obtained from MAGICS.[E1,E2] All the control subjects (n = 694) and 73 asthmatic cases were from the large (n = 5629) cross-sectional ISAAC II[E1,E4] from the same ethnic population source. Because of subsequent quality controls and an update of consent in a follow-up study, the number of subjects for whom the chip genotyping results were available for the purposes of the present study decreased to 1303 (n = 651 asthmatic patients). For matrix-assisted laser desorption ionization time-of-flight mass spectrometric genotyping, 1454 subjects were available: those from chip genotyping in the asthma project (n = 1303), children recruited later in the study (n = 30), children not included in the previous asthma analysis because of insufficient DNA for chip genotyping (n = 113), and unconfirmed asthma status (n = 8). Samples with unconfirmed asthma status were not included in the association analyses, leading to a total of 1446 subjects.

Between 1995 and 1996, a cross-sectional study was conducted in Germany (Munich: n = 1159, Dresden: n = 1940 [ISAAC II],[E4] and Leipzig: n = 1165[E5]; see Tables E1 and E2) to assess the prevalence of asthma and allergies in schoolchildren (9-11 years). Children were classified as having asthma if parents reported a physician's diagnosis of asthma at least once or if spastic or asthmatic bronchitis was reported more than once in self-administered questionnaires. A child was considered atopic with a wheal reaction of 3 mm or larger after skin prick tests (SPTs) to 1 or more of 6 common allergens after subtraction of the negative control.[E4,E5] Atopic asthma was defined as the concomitant occurrence of asthma and a positive SPT response. Nonatopic asthmatic patients included asthmatic patients without positive SPT responses. Both atopic and nonatopic asthmatic patients were compared with a reference group including nonasthmatic subjects without positive SPT responses (further referred to as nonatopic nonasthmatic subjects).

From 2001 to 2007, children (mean age, 11 years) in 7 German and Austrian clinical asthma centers in Bochum, Cologne, Feldkirch, Freiburg, Munich/Rosenheim, Vienna, and Wesel were recruited for MAGICS. A pediatric pulmonologist diagnosed asthma and allergy according to clinical guidelines and objective measures, such as results lung function tests, clinical examination, history, and allergy testing.[E2,E6] Sensitization to common inhalant allergens, such as *D pteronyssinus*, *Dermatophagoides farinae*, cat epithelium, grass pollen, and birch pollen, was assessed based on measurement of specific serum IgE levels with CLA-1 (Hitachi Chemical Diagnostics, Mountain View, Calif) or AllergyScreen (Mediwiss Analytic, Moers, Germany) panels.[E6] In ISAAC II, as part of this case-control population, atopic sensitization was tested by using SPTs to *D pteronyssinus*, *D farinae*, *Alternaria tenuis*, cat dander, mixed grass and mixed tree pollen, and/or a Sx1 panel (ImmunoCAP, Phadia, Germany), covering a mix of the following allergens: local grass pollen, rye pollen, birch pollen, mugwort pollen, *D pteronyssinus*, cat dander, dog dander, and *Cladosporium herbarum*. Nonatopic asthma was stringently specified as asthma and no sensitization (RAST class equivalent of 0). Both atopic and nonatopic asthmatic patients were compared with a reference group including nonasthmatic subjects without sensitization (further referred to as nonatopic nonasthmatic subjects).

All included study subjects of both populations (ISAAC II and MAGICS) were of German descent. Written informed consent was obtained from all parents of children included in these studies. Study methods that were very similar in both populations were approved by the respective ethics committees.

EMSA

Nuclear extract was prepared from 14×10^6 Jurkat T, HEK293, and YT cells obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany), either unstimulated or stimulated with PMA (50 ng/mL)/ionomycin (1 μmol/L) or *D pteronyssinus* (Der p 1, 30 μg/μL; Indoor Biotechnologies, Warminster, United Kingdom) for 3 hours. PBMCs were isolated by means of density gradient centrifugation with Ficoll-Hypaque[E7] and either left unstimulated or stimulated with PMA (50 ng/mL)/ionomycin (1 μmol/L) or Der p 1 (30 μg/μL, Indoor Biotechnologies) for 3 hours. Cells were resuspended in 120 μL of buffer A (10 mmol/L 4-HEPES, 3 mmol/L $MgCl_2$, 40 mmol/L KCl, 1 mmol/L dithiothreitol, 5% glycerol, and 0.2% NP-40) supplemented with protease (1 mmol/L phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL antipain, and 10 μg/mL pepstatin) and phosphatase inhibitors (1 mmol/L benzamidine, 1 mmol/L orthosodium vanadate, 1 mmol/L sodium fluoride, and 5 mmol/L β-glycerophosphate). Sedimented nuclei were resuspended in Buffer C (20 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 1 mmol/L dithiothreitol, and 25% glycerol supplemented with protease and phosphatase inhibitors, as in Buffer A) in corresponding volumes.[E7] Nuclear extracts were placed in aliquots and stored at −80°C. Protein concentration of nuclear extract was determined by using the BCA kit (Thermo Fisher Scientific, Uppsala, Sweden). Annealing of the complementary oligonucleotide pairs was carried out in 10 mmol/L Tris Cl (pH 8), 1 mmol/L EDTA, and 100 mmol/L NaCl by boiling the samples for 5 minutes and cooling slowly to 37°C. Purified double-stranded probes corresponding to the risk or nonrisk allele of rs8076131 (33 bp, 100 ng) or rs4065275 (31 bp, 100 ng) were end-labeled by using $\gamma[^{32}P]$-ATP (250 μCi) with T4 polynucleotide kinase (New England Biolabs GmbH, Frankfurt am Main, Germany). Free radioactivity was removed by using the mini Quick Spin Oligo Columns (Roche GmbH, Mannheim, Germany). For EMSA experiments, each binding reaction (20 μL) contained 5 μg of nuclear extract, 1× binding buffer (10 mmol/L Tris Cl [pH = 8], 1 mmol/L EDTA, and 0.1 mmol/L β-mercaptoethanol), and 0.25 μg per reaction of Poly(dI-dC)-Poly(dI-dC) and was adjusted for a final concentration of 80 mmol/L NaCl and 4% glycerol. Supershift experiments for the transcription factors USF1, USF2, SP1, SP3, and SP4 (Santa Cruz Biotechnology, Santa Cruz, Calif) were performed by means of preincubation with antibody (4 μg) for 30 minutes on ice. The respective ^{32}P-labeled protein/DNA complexes were resolved on a 5% polyacrylamide gel. Consensus sequences for the transcription factors USF[E8] and SPs[E9] were used for competition experiments (100-fold molar excess).

Luciferase promoter activity assay

HEK293 cells were seeded in a 24-well plate at a density of 1.3×10^5 cells per well. The next day, cells were transfected with 250 ng of pGL3 plasmids expressing a luciferase gene under the control of the *ORMDL3* promoter (1069 bp) containing either both risk alleles of rs8076131 and rs4065275, a combination of risk and nonrisk alleles, or the nonrisk allele of each SNP. Site-directed mutagenesis of pGL3-*ORMDL3* constructs was performed to replace the risk alleles rs8076131 and rs4065275 by their respective nonrisk alleles separately or with a combination of both nonrisk alleles (Agilent Technologies, Santa Clara, Calif). The sequence of *ORMDL3* promoters was confirmed by means of resequencing. In total, 4 pGL3-*ORMDL3* (250 ng) constructs were transiently transfected. Ten nanograms of pRL-TK *Renilla* reporter plasmid (Promega, Madison, Wis) was cotransfected for normalization of transfection efficiency and cell viability. Lipofectamin 2000 was used as the transfection reagent, according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Cells were left in medium or stimulated with 0.1 μmol/L ionomycin, 1 μmol/L ionomycin, or 50 ng/mL PMA plus 1 μmol/L ionomycin 3 hours after transfection and harvested 24 hours after transfection. Cells were washed in PBS and lysed in 1× passive lysis buffer (Promega). A dual-luciferase assay was performed, according to the manufacturer's protocol (Promega), by using Synergy HT (BioTek, Winooski, Vt) and quantified as relative light units. Experiments were conducted independently 5 times, with 3 technical replicates for each construct.

Allele-specific *ORMDL3* mRNA expression and cytokine analyses in PBMCs in unstimulated and stimulated samples

Allele-dependent effects of the *ORMDL3* promoter polymorphisms rs8076131 and rs4065275 on *ORMDL3* gene expression were analyzed in PBMCs from EXACT (January 2012-March 2013, Hannover, Germany) by

using real-time PCR. Adults of German origin (n = 61; 28 male subjects; mean age, 32.5 ± 8.6 years) were recruited. Questionnaires addressed whether participants had 1 or more of the following immunologic disorders: asthma, hay fever, atopic dermatitis (atopic eczema and endogenous eczema), psoriasis, diabetes mellitus, rheumatoid disease, ulcerous colitis, Crohn disease, multiple sclerosis, or any other autoimmune disease. In addition, time of disease onset and current persistence, SPT results, smoking status (former or current), and medications taken within a week before sample collection were documented for all study subjects. Written informed consent was obtained from all participants, and the study protocol was approved by the Hannover Medical School Ethics Committee.

For the purposes of the current project, sufficient RNA from unstimulated PBMCs and PBMCs stimulated with all 4 stimuli (Der p 1, LpA, Ppg, and PHA) were obtained from 57 adults. Four subjects were excluded from the analyses because they did not pass all our stringent quantitative real-time PCR quality control criteria. An additional 5 subjects were excluded because of their reported asthma status in the questionnaire. Thus allele-specific gene expression analyses for *ORMDL3* in PBMCs included 47 adult nonasthmatic subjects.

Fifty milliliters of venous blood was obtained from each subject. DNA was isolated from whole blood, according to the manufacturer's protocol (QIAAmp Blood Isolation Kit; Qiagen, Hilden, Germany). PBMCs were isolated by means of density gradient centrifugation with Ficoll-Hypaque[E7] and stimulated with natural *D pteronyssinus* (Der p 1, 30 μg/μL, Indoor Biotechnologies), LpA (0.1 μg/μL, Sigma-Aldrich, Steinheim, Germany), Ppg (10 μg/μL, Sigma-Aldrich), and PHA (5 μg/μL, Sigma-Aldrich) for 48 hours and compared with that in unstimulated cells. Supernatants of respective samples were collected and stored at −80°C for cytokine measurements. For the purposes of the present study, cytokine levels were measured in supernatants derived from 54 nonasthmatic subjects. Total RNA from PBMCs was isolated with the NucleoSpin RNAII kit (Macherey-Nagel, Dueren, Germany), according to the manufacturer's instructions. Reverse transcription of total RNA (1 μg) was conducted with QuantiTect Reverse Transcription (Qiagen). Genomic DNA from whole blood was extracted with the FlexiGene DNA kit (Qiagen), according to the manufacturer's recommendations. Specific primers and probes for *ORMDL3* and the housekeeping gene *18SrRNA* were designed with Vector NTI Advance 10 software (Invitrogen, Life technologies, Carlsbad, Calif). 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 *ORMDL3* and the housekeeping gene *18SrRNA* was used to calculate ΔCt. Relative fold changes for the cohort were calculated by using the $2^{-\Delta\Delta Ct}$ algorithm according to Livak and Schmittgen.[E10] Statistical analyses were performed with SigmaStat3.5 and R software (Version 3.0.0).[E11]

Genotyping of rs8076131 and rs4065275 in the EXACT study population

The rs8076131 polymorphism was genotyped by means of matrix-assisted laser desorption ionization time-of-flight mass spectrometry.[E12] The genotype of rs4065275 was determined by using restriction fragment length polymorphism in 61 subjects from the EXACT cohort. PCR primers (forward: 5′ TGAGACACAGTCCAGCAGGAGT 3′, reverse: 5′ CAGGCTGCCCCATGAATC 3′; Metabion International, Martinsried, Germany) were designed, introducing a mismatch site for *Hinf*I restriction enzyme (10,000 U/mL supported with 10× CutSmart Buffer; New England Biolabs, Ipswich, Mass). The PCR reaction was carried in 15 μL containing 1.5 μL of 10× ThermoPol Buffer (New England Biolabs), 0.05 μL of Taq Polymerase (New England Biolabs), 0.3 μL of deoxynucleotide triphosphates (10 mmol/L, peqGold dNTP-Set; PeqLab Biotechnologie GmbH, Erlangen, Germany), 0.18 μL of forward and reverse primer (25 pmol/μL each), 1.5 μL of Betain (5 mol/L to a final concentration of 0.5 Mmol/L, Sigma-Aldrich), 2 μL of genomic DNA (20 ng/μL), and 9.29 μL of water (BioScience Grade, nuclease free, sterile filtered, and steam sterilized for molecular biology, DEPC treated; Carl Roth, Karlsruhe, Germany). The following PCR cycling conditions were applied: 94°C for 2 minutes of denaturation, 40 × 94°C, 59.7°C for 20 seconds, 72°C for 30 seconds, and 72°C for 7 minutes (final elongation). The PCR products were subjected to enzymatic digestion and the mixture for a single reaction of 20 μL containing 1.5 μL of *Hinf*I restriction enzyme, 2 μL of 10× CutSmart buffer, 11.5 μL water, and 5 μL of PCR product.

Incubation at 37°C was performed for 60 minutes, followed by 30 minutes of enzyme deactivation at 80°C. Digested DNA fragments were then loaded on a 3.5% agarose gel prelabeled with GelRed Nucleic Acid Stain (Biotium, Hayward, Calif). The PCR fragment (208 bp) after digestion led to the following pattern: homozygote risk alleles (GG) of 14 bp, 19 bp, and 175 bp; heterozygote alleles (GA) of 14 bp, 19 bp, 175 bp, and 194 bp; and homozygote nonrisk alleles (AA) of 14 bp and 194 bp.

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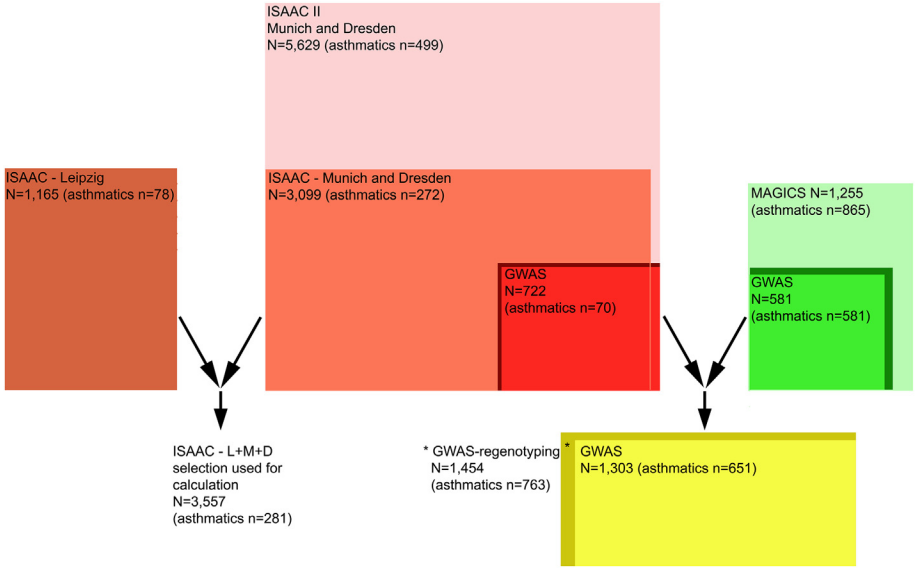

FIG E1. Study design and population selection. The cross-sectional ISAAC study population consists of samples from Munich, Dresden (ISAAC II), and Leipzig. A reference for the GWAS was additionally drawn from the Munich and Dresden population. To avoid any overlap, the samples selected for GWASs were excluded from all analyses within the cross-sectional cohort. *Bright red* and *green* colors indicate samples combined for the GWAS analysis *(bright yellow)*. Darker borders depict the initially selected samples that did not pass all quality control for chip genotyping. Therefore more samples were available for regenotyping *(dark yellow)* than for GWAS analyses.

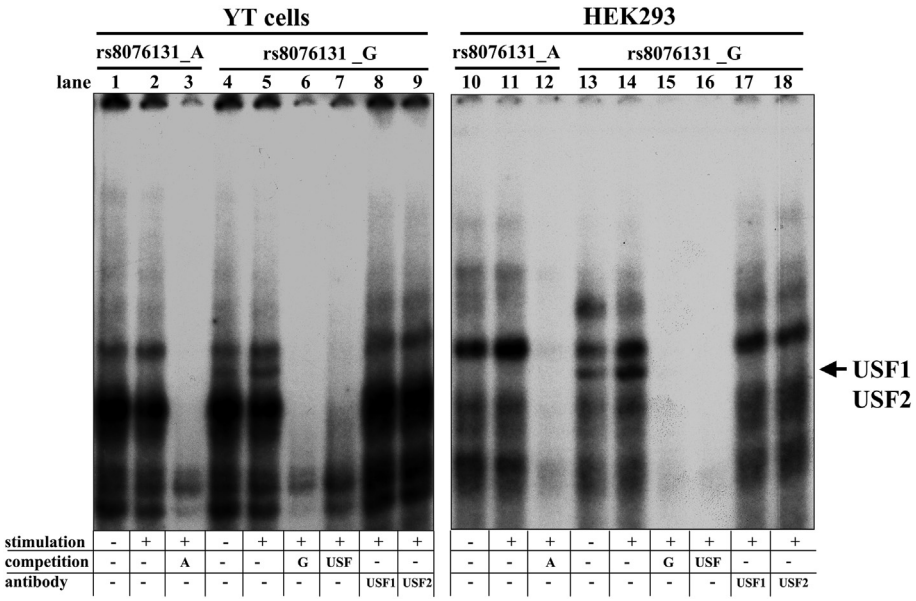

FIG E2. SNP rs8076131 in an *ORMDL3* promoter region induces novel binding sites for USF1 and USF2 in YT and HEK293 cells. EMSA analysis with an *ORMDL3* probe either carrying the risk A (rs8076131_A, *lanes 1-3* and *10-12*) or nonrisk G (rs8076131_G, *lanes 4-9* and *13-18*) allele of rs8076131 was performed with nuclear extract from YT *(lanes 1-9)* and HEK293 *(lanes 10-18)* cells (5 μg) cultured for 3 hours and either left in medium *(−)* or stimulated *(+)* with PMA/ionomycin (50 ng/mL and 1 μmol/L, respectively). Competitors (100-fold molar excess) and supershift antibodies (4 μg) for each experiment are noted below the respective lanes.

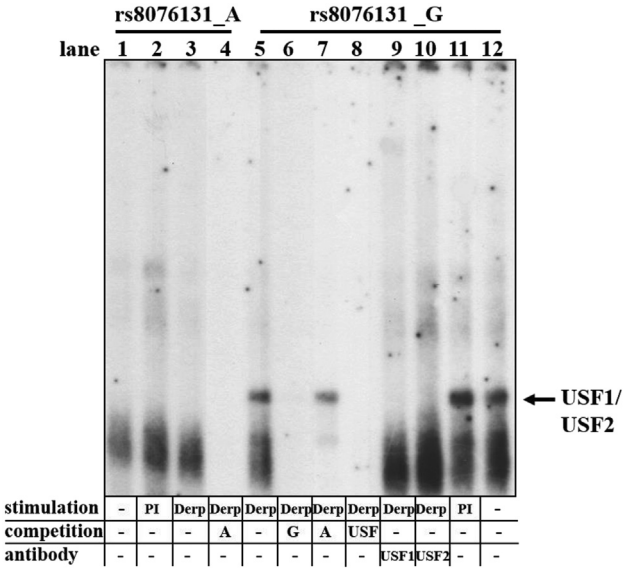

FIG E3. SNP rs8076131 in an *ORMDL3* promoter region induces novel binding sites for USF1 and USF2 after stimulation with Der p 1. EMSA analysis with an *ORMDL3* probe either carrying the risk A (rs8076131_A, *lanes 1-4*) or the nonrisk G (rs8076131_G, *lanes 5-12*) allele of rs8076131 was performed with nuclear extract from Jurkat T cells (5 μg) cultured for 3 hours and either left in medium *(−)* or stimulated *(+)* with PMA/ionomycin (50 ng/mL and 1 μmol/L, respectively) or Der p 1 (30 μg/μL). Competitors (100-fold molar excess) and supershift antibodies (4 μg) for each experiment are noted below the respective lanes.

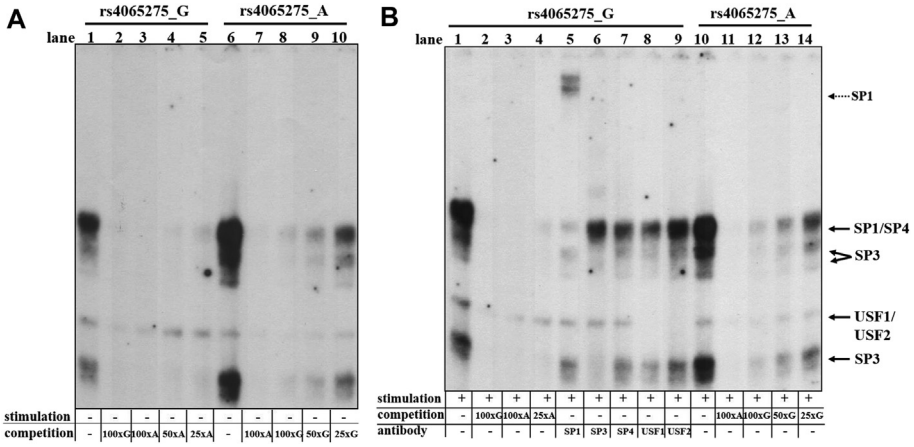

FIG E4. SNP rs4065275 in an *ORMDL3* promoter region revealed allele-specific affinity binding differences of the SP transcription factor. **A,** EMSA analysis with an *ORMDL3* probe either carrying the risk G (rs4065275_G, *lanes 1-5*) or the nonrisk A (rs4065275_A, *lanes 6-10*) allele of rs4065275 was performed with nuclear extract from the Jurkat T cells (5 μg) cultured for 3 hours in medium. Competitors (100-, 50-, or 25-fold molar excess) for each experiment are noted below the respective lanes. **B,** EMSA analysis with an *ORMDL3* probe either carrying the risk G (rs4065275_G, *lanes 1-9*) or nonrisk A (rs4065275_A, *lanes 10-14*) allele of rs4065275 was performed with nuclear extract from the Jurkat T cells (5 μg) stimulated for 3 hours with Der p 1 (30 μg/μL). Competitors (100-, 50-, or 25-fold molar excess) and supershift antibodies (4 μg) for each experiment are noted below the respective lanes.

TABLE E1. Population characteristics of the cross-sectional study population (ISAAC II)

	Genotyped study population (n = 4264)		Study population without GWAS samples (n = 3557)	
	No.	Percent	No.	Percent
Male sex	2151/4257	50.53	1785/3550	50.28
Age*	9.7 ± 0.60		9.72 ± 0.61	
Asthma	350/4185	8.36	281/3484	8.07
SPT reactivity	1056/4110	25.69	873/3430	25.45
Hay fever (doctor's diagnosis)	336/4174	8.05	259/3484	7.43
Hay fever (doctor's diagnosis + SPT)	252/4025	6.26	187/3512	5.32
Bronchial hyperresponsiveness	300/2468	12.16	232/2148	10.80
Atopic dermatitis†	540/2994	18.04	416/2314	17.98
Eczema‡	679/3960	17.15	555/3280	16.92

*Mean ± SD.
†Because a doctor's diagnosis of atopic dermatitis is not available in Leipzig, atopic dermatitis is only reported for children from Dresden and Munich.
‡Eczema is defined by a combination of reporting as "itchy dermal changes" and "ever eczema" in Leipzig children and atopic dermatitis in Munich or Dresden children.

TABLE E2. Population characteristics of the case-control study population (MAGICS/ISAAC II)

	MAGICS (n = 691)		ISAAC (n = 763)	
	No.	Percent	No.	Percent
Male sex	457/691	66.14	386/763	50.59
Age*	10.99 (2.90)		9.63 (0.59)	
Asthma	691/691	100	72/755	9.54
Atopic sensitization	505/598	84.45	194/651	29.80

*Mean (SD).

TABLE E3. Description and quality control for genotyped SNPs of the 17q21 locus and *ORMDL3* in the cross-sectional study population (n = 3557)

Genotyped SNP	Nonrisk allele	Risk allele	AF risk allele†	Call rate	Bins within 17q21	r^2 with tag SNP	*P* value (HWE)
rs9303277	T	C	0.49	93.6%	1	0.83	.7816
rs11557467	T	G	0.51	93.4%	1	0.85	.3855
rs8067378	G	A	0.49	95.7%	1	0.86	.1704
rs2290400	G	A	0.51	92.9%	1	0.89	.2102
rs7216389	C	T	0.49	93.8%	1	0.91	.9444
rs3169572*	G	A	0.04	95.3%			.3084
rs4065275*	A	G	0.52	95.4%	1		.1393
rs8076131*	G	A	0.55	95.7%	2		.4883
rs17608925*	C	T	0.90	95.0%			.0751
rs3744246*	T	C	0.82	95.2%			.1858
rs4795402*	A	C	0.77	95.5%			.4088
rs4795405	T	C	0.57	93.4%	2	0.91	.9156
rs8079416	T	C	0.54	94.3%	3		.4865
rs4795408	G	A	0.55	93.6%	3	0.96	.9442
rs3894194	C	T	0.54	94.4%	3	0.91	.7022
rs3859192	C	T	0.45	93.5%			.6761

HWE, Hardy-Weinberg equilibrium (*P* value of χ^2 test for deviation of HWE).

*Boldface letters represent the identified tagging SNPs for *ORMDL3* based on an r^2 value of 0.85 or greater.

†Because allele frequencies *(AF)* for most SNPs on 17q21 were close to 50%, the respective risk allele (instead of the minor allele) is depicted, which was defined as the allele increasing the risk for asthma development.

TABLE E4. Description and quality control for genotyped SNPs of the 17q21 locus and *ORMDL3* in the case-control study population (n = 1454)

Genotyped SNP	Nonrisk allele	Risk allele	AF risk allele*	Call rate	*P* value (HWE)
rs3169572	A	G	0.96	92.0%	.0989
rs4065275	A	G	0.54	94.8%	.5162
rs8076131	G	A	0.57	95.8%	.0492
rs17608925	C	T	0.91	91.7%	.1860
rs3744246	T	C	0.82	98.6%	.1258
rs4795402	A	C	0.78	99.1%	.8195

HWE, Hardy-Weinberg equilibrium (*P* value of χ^2 test for deviation of HWE).
*Because allele frequencies *(AF)* for some *ORMDL3* alleles were close to 50%, the respective risk allele (instead of the minor allele) is depicted, which was defined as the allele increasing the risk for asthma development.

TABLE E5. Single-stranded oligonucleotides used for allele-specific transcription factor binding analyses depending on rs8076131 and rs4065275

Probe name	Probe sequence (5′3′)
rs8076131_T_fwd	ATGGGAAGAGCTTAAGAGTAGATGTCCCACATT
rs8076131_T_rev	AATGTGGGACATCTACTCTTAAGCTCTTCCCAT
rs8076131_C_fwd	ATGGGAAGAGCTTAAGAGCAGATGTCCCACATT
rs8076131_C_rev	AATGTGGGACATCTGCTCTTAAGCTCTTCCCAT
rs4065275_C_fwd	CAGTCCAGCAGGGGTCGTGCTGAGTGAGAAT
rs4065275_C_rev	ATTCTCACTCAGCACGACCCCTGCTGGACTG
rs4065275_T_fwd	CAGTCCAGCAGGGGTTGTGCTGAGTGAGAAT
rs4065275_T_rev	ATTCTCACTCAGCACAACCCCTGCTGGACTG
USF consensus sequence_fwd[E8]	CACCCGGTCACGTGGCCTACACC
USF consensus sequence_rev[E8]	GGTGTAGGCCACGTGACCGGGTG
SP consensus sequence_fwd[E9]	ATTCGATCGGGGCGGGGCGAGC
SP consensus sequence_rev[E9]	GCTCGCCCCGCCCCGATCGAAT

TABLE E6. Minimal and maximal detection limit of IL-4 and IL-13 extrapolated from a 9-point standard curve

		Detection limits (pg/mL)	
Cytokine	**Stimuli**	**Minimal**	**Maximal**
IL-4	Unstimulated	0.21	4890.60
	Der p 1	0.24	4592.93
	LpA	0.26	4436.50
	Ppg	0.22	4555.93
	PHA	0.05	3827.84
IL-13	Unstimulated	0.58	28446.33
	Der p 1	0.58	9988.31
	LpA	0.51	10219.16
	Ppg	0.61	9587.58
	PHA	0.55	997368
IL-8	Unstimulated	0.38	27568.17
	Der p 1	1.67	26775.08
	LpA	6.11	23072.99
	Ppg	6.47	33612.66
	PHA	0.44	28177.04

TABLE E7. Tagging bins within the *ORMDL3* region (5 kb upstream and downstream) based on HapMap and the 1000 Genomes Project data set

Tagging bin	SNP	Position (chromosome 17)	MAF	Alleles (major/minor)
1	rs78199107	38,077,750	0.035	T/C
	rs3859186	38,076,329	0.035	G/A
	rs3169574	38,077,485	0.035	G/A
	rs3169572	38,077,412	0.035	G/A
	rs145604770	38,086,736	0.035	G/A
	rs113894104	38,072,955	0.035	C/T
	rs112599791	38,072,475	0.035	G/A
	rs112260932	38,077,185	0.035	T/C
	rs112191651	38,088,150	0.035	C/T
	rs111379006	38,075,100	0.035	G/A
2	rs9303281	38,074,046	0.494	A/G
	rs9303280	38,074,031	0.476	C/T
	rs8074437	38,076,137	0.494	G/T
	rs8065777	38,072,402	0.476	T/C
	rs7224129	38,075,426	0.494	A/G
	rs7219923	38,074,518	0.494	T/C
	rs4065275	38,080,865	0.465	G/A
	rs12603332	38,082,807	0.465	C/T
3	rs9303279	38,073,968	0.459	C/G
	rs8076131	38,080,912	0.441	A/G
	rs4795405	38,088,417	0.429	C/T
	rs2872516	38,072,727	0.465	T/C
	rs17608925	38,082,831	0.129	T/C
4	rs7224908	38,086,854	0.212	G/A
	rs4795404	38,085,791	0.212	C/A
	rs4795403	38,085,722	0.212	C/T
	rs3744246	38,084,350	0.212	C/T
	rs12946393	38,087,429	0.212	G/T
	rs4795402	38,085,385	0.247	C/A
	rs35557848	38,087,439	0.218	C/T
	rs111935829	38,081,394	0.053	C/G

Boldface letters represent the identified tagging SNPs for *ORMDL3* based on an r^2 value of 0.85 or greater.
MAF, Minor allele frequency.

TABLE E8. Transcription factor binding patterns predicted by means of *in silico* analyses assessed for the *ORMDL3* tagging and single SNPs

Bin	SNP	Location	Nonrisk allele	Diff. TF binding to nonrisk allele*	Risk allele†	Diff. TF binding to risk allele*	FS score*
1	rs3169572	3′ UTR ORMDL3/promoter GSDMB	A		G		0.33
2	rs4065275	Intron 2/alternative promoter	A		G	CF1, Ttk 69	0.5
3	rs8076131	Intron 2/alternative promoter	G	E47, GATA-2, MyoD	A		0.5
	rs17608925	Intron 2	C	ATF, CREB	T	GATA-1, AML-1a, MATa1	0.716
4	rs3744246	Promoter	T		C		
	rs4795402	Promoter	A		C		0.398
	rs35557848	Upstream	C		T		
	rs111935829*	Intron 2	G		C		

FS score, Functional significance score; *TF*, transcription factor.
*Allele-specific prediction for changes of transcription factor binding based on F-SNP software (http://compbio.cs.queensu.ca/F-SNP/).
†Risk allele (instead of the minor allele) is depicted, which was defined as the allele increasing the risk for asthma development.