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17q12-21 risk-variants influence cord blood immune regulation and multitrigger-wheeze

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Abstract

Background: Childhood wheeze represents a first symptom of asthma. Early identification of children at risk for wheeze related to 17q12-21 variants and their underlying immunological mechanisms remain unknown. We aimed to assess the influence of 17q12-21 variants and mRNA expression at birth on the development of wheeze.

Methods: Children were classified as multitrigger/viral/no wheeze until six years of age. The PAULINA/PAULCHEN birth cohorts were genotyped (n = 216; GSA-chip). mRNA expression of 17q21 and innate/adaptive genes was measured (qRT-PCR) in cord blood mononuclear cells. Expression quantitative trait loci (eQTL) and mediation analyses were performed. Genetic variation of 17q12-21 asthma-single nucleotide polymorphisms (SNPs) was summarized as the first principal component (PC1) and used to classify single SNP effects on gene expression as (locus)-dependent/independent eQTL SNPs.

Results: Core region risk variants (*IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3*) were associated with multitrigger wheeze (OR: 3.05–5.43) and were locus-dependent eQTL SNPs with higher *GSDMA*, *TLR2*, *TLR5*, and lower *TGFB1* expression. Increased risk of multitrigger

Abbreviations: CASP1, Caspase 1; CBMCs, cord blood mononuclear cells; CD, cluster of differentiation; Chr, chromosome; CI, confidence interval; CT, cycle threshold; DCs, dendritic cells; eQTL, expression quantitative trait loci; ERBB2, Erb-B2 receptor tyrosine kinase 2; ERP, European Reference Population; FU, follow-up; GSA, global screening array; GSDMA/B, Gasdermin A/B, alias GSDM1/L; HWE, Hardy-Weinberg equilibrium; IFIH1, interferon-induced with helicase C domain 1, alias MDA-5; IKZF3, IKAROS family zinc finger 3, alias ZNFN1A3; IL, interleukin; IPS1, IFN-beta promoter stimulator-1, alias MAVS; LD, linkage disequilibrium; LpA, lipid A; LY75, lymphocyte antigen 75, alias CD205; MTW, multitrigger wheeze; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3; NW, no wheeze; OR, odds ratio; ORMDL3, ORM1-like 3; PAULINA/PAULCHEN, Pediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies/Prospective Cord Blood Study in Rural Southern Germany; PC1, first principal component; PCA, principal component analysis; PGAP3, post-GPI attachment to proteins phospholipase 3; PHA, phytohemagglutinin; qRT-PCR, quantitative real-time polymerase chain reaction; RAF, risk allele frequency; RORA/C3/C4, RAR-related orphan receptor A/C3/C4; SNP, single nucleotide polymorphism; TGFB1, transforming growth factor-beta 1; Th2, T helper type 2; TLR (2/4/5/7), Toll-like receptor (2/4/5/7); TNFAIP3, tumor necrosis factor (TNF) alpha-induced protein 3, alias A20; TNIP2, TNFAIP3-interacting protein 2; TRAF6, TNF receptor-associated factor 6; U, unstimulated; VIF, variance inflation factor; VW, viral wheeze; ZPBP2, zona pellucida-binding protein 2.

wheeze with rs9303277 was in part mediated by *TLR2* expression. Risk variants distal to the core region were mainly locus-independent eQTL SNPs with decreased *CD209*, *CD86*, *TRAF6*, *RORA*, and *IL-9* expression. Distinct immune signatures in cord blood were associated either with multitrigger wheeze (increased innate genes, e.g., *TLR2*, *IPS1*, *LY75*) or viral wheeze (decreased NF-κB genes, e.g., *TNFAIP3* and *TNIP2*).

Conclusion: Locus-dependent eQTL SNPs (core region) associated with increased inflammatory genes (primarily *TLR2*) at birth and subsequent multitrigger wheeze indicate that early priming and imbalance may be crucial for asthma pathophysiology. Locus-independent eQTL SNPs (mainly distal region, rs1007654) may be involved in the initiation of dendritic cell activation/maturation (*TRAF6*) and interaction with T cells (*CD209*, *CD86*). Identifying potential mechanistic pathways at birth may point to critical key points during early immune development predisposing to asthma.

KEYWORDS
childhood wheeze and asthma, chromosome 17, cord blood, eQTL, mediation analyses, mRNA expression, single nucleotide polymorphisms

1 | INTRODUCTION

Asthma remains the most common chronic disease in children worldwide with increasing prevalence.[1] First symptoms develop before age six, including wheeze. Previously, wheezing trigger (multitrigger/viral) and pattern over time (transient/late/persistent) were described in young children.[2] However, only one-third will continue to have persistent/recurrent symptoms and develop asthma. Currently, limited clinical and diagnostic criteria exist for risk evaluation of recurrent wheeze considering complex interactions between genetics, environment, and immune regulation.

One valuable tool for early risk assessment is common genetic variants as predisposing factors. Chromosome (chr) 17q12-21 is the most replicated asthma susceptibility locus, with risk variants strongly associated with childhood-onset but not adult-onset asthma[3–5] and further with early-life wheezing.[6,7] Also, proximal (*PGAP3*, *ERBB2*), core (*IKZF3*, *ZPBP2*, *GSDMB*, *ORMDL3*), and distal regions (*GSDMA*) of 17q12-21 locus were suggested as independent asthma-associated loci.[8,9] As most 17q12-21 risk variants are non-coding, their functional relevance has not been fully characterized yet. Different expression quantitative trait loci (eQTL) studies showed associations of 17q12-21 risk variants with *ORMDL3*, *GSDMA*, and *GSDMB* (*cis*-regulatory effects),[10,11] that regulate different molecules (*ICAM-1*, *CD49d*, *CD18*, *CD48*),[12,13] being correlated with T-cell-associated cytokines (IL-2, IL-4, IL-6, IL-13, IL-17, TNF).[10,11,14] However, underlying immunological mechanisms leading to early-life wheezing as the first clinical manifestation of childhood asthma remain unsolved.

In our longitudinal PAULINA/PAULCHEN birth cohorts, we investigated the impact of risk variants on wheeze. Expression patterns of 17q21 and innate/adaptive genes in cord blood mononuclear cells (CBMCs) were examined concerning 17q12-21 risk variants and disease phenotypes, followed by mediation analyses.

Key message

Our genetic findings identify an association between wheeze risk and genes involved in inflammation and dendritic cell maturation/activation. This novel finding points to key regulatory mechanisms of how 17q12-21 risk variants can regulate immune responses already at birth, which may contribute to asthma susceptibility later in life. Identification of these immunoregulatory mechanisms is critical, as underlying mechanisms of the strongest known genetic risk for childhood asthma were present already at birth. The hereby identified results may pave the ground for future functional studies, prediction of disease development, and the development of targeted asthma treatments.

2 | METHODS

Details in Supporting-Information.

2.1 | Study population

Healthy pregnant women with uncomplicated pregnancies and deliveries ($n = 283$) from Munich and outer area were recruited for two birth cohorts PAULINA[15] (enriched for maternal atopy, $n = 190$) and PAULCHEN[16] (rural areas, $n = 93$) during the last pregnancy trimester (2004–2008). The subpopulation under study (with complete genotype and wheeze phenotype data; $n = 180$) was representative compared with the whole cohort with respect to sex, birth mode, gestational age, maternal smoking, atopy, education, and paternal

atopy, showing no significant differences. Written informed consent was obtained from parents. The human ethics committee (Bavarian Ethical Board; LMU Munich #04092) approved the study. Data were collected by questionnaires completed by mothers/fathers for unrelated children after birth and at follow-ups (3,6,10 years; FU3/6/10, n = shown in Figure S1).

2.2 | Genotyping

DNA samples were extracted from EDTA cord blood collected at birth, quality measured with Bioanalyzer, and single nucleotide polymorphisms (SNPs) genotyped with Infinium Global Screening Array v2.0 (GSA). Phasing and imputation for variants with a minor allele frequency >1% were conducted with the cosmopolitan reference panel 1000 Genomes Phase 3 Project (European Reference) using RESHAPE and IMPUTE2 software.[17–19] Genotyping quality was ensured by plausibility checks for sex and allele frequencies (subject- and SNP-wise call rates >95%) and deviation from Hardy-Weinberg equilibrium (HWE, Chi-square test, $p > .05$; laboratory materials, Table S1).

2.3 | Isolation of CBMCs and quantitative RT-PCR

CBMCs were isolated after blood withdrawal from the umbilical vein and were either kept unstimulated (U) or stimulated for 72 h with phytohemagglutinin (PHA: 5 μg/ml) or lipid A (LpA: 0.1 μg/ml). After cell harvest and mRNA extraction, in-depth quality control steps were performed, and mRNA expression was assessed (qRT-PCR) in a subgroup at birth as previously described (Supporting-Information).[15,16] Gene selection was based on current literature being central for the pathogenesis of allergic and inflammatory diseases (innate/adaptive immunity; Table S2).

2.4 | Definition of variables

Outcomes included wheeze phenotypes and patterns over time, any allergy, and parent-reported doctor's diagnosis of atopic dermatitis, hay fever, and asthma (Supporting-Information). *Wheeze* (FU3/6) was parent-reported. *Viral wheeze* (VW) was defined by wheezing only during acute (viral) infection, *multitrigger wheeze* (MTW) by having wheezing episodes outside of infection with unspecific triggers and/or by ≥2 defined triggers causing wheeze (with infection, effort, excitement, house dust, animals, grasses), and *no wheeze* (NW) by having no respiratory symptoms at any age.

2.5 | SNP selection

Asthma-associated 17q12-21 SNPs (chr17: 37,814,080 – 38,197,914) with $p < 1e\text{-}8$ (n = 23) were chosen, based on associations with childhood-onset asthma by Moffatt et al.,[3] replicated in recent studies[4,5,20] and shown to be relevant for potential biological mechanisms.[21-24] Pairwise linkage disequilibrium (LD) was derived from our cohort and European Reference Population (ERP) using the online tool "*LDlink!*". We reported findings for n = 12 SNPs, being in high LD ($r^2 > .80$) for a block of SNPs with the lowest *p*-value[3] or remaining as single SNP not connected to a block.

2.6 | Statistical analyses

Data analyses and visualization were performed using R (Supporting-Information).

2.6.1 | Multiple testing

Multiple testing was addressed by correcting for the effective data dimension. *SNP data:* eigenvalue decomposition of LD matrix revealed that the first seven eigenvectors explained > 95% of the LD structure (Figure S2A). Therefore, dimension n = 7 was used as a correction factor for SNP data and $p \leq .0071$ (=.05/7) was reported as significant. *Gene expression data:* hierarchical clustering using gene-gene correlation was performed (single-linkage). The cutoff r = .63 was derived by assessing the distribution under the assumption of no-correlation structure as a reference (90% quantile, Figure S2B)[25], corresponding to $n = 5$ clusters (Figure S2C). Therefore, n = 5 was used as a correction factor for gene expression and $p \leq .01$ (=.05/5) was reported as significant.

2.6.2 | Association analyses

The first principal component (PC1) of 23 SNPs explaining 60% of the variance was built to summarize the overall genetic variation and used as (adjustment) covariate (Figure S2D). High LD leading to redundant information was taken into account by the PC analysis (PCA). First, we examined genotype-phenotype associations (*analysis a*) followed by genotype-gene expression associations (*analysis b*). Gene expression-phenotype associations were investigated in *analysis c*. To quantify the effect of genotype on wheeze via gene expression, causal mediation analyses were performed (*analysis d*). Correlation analyses of gene expressions were performed (Spearman rank correlation). For evaluation of potential confounders, differences between phenotypes were assessed (Kruskal-Wallis or Chi-square test). To avoid overadjustment, we did not consider wheeze-related atopic variables such as asthma or hay fever as control variables.

Genotype-Phenotype Association (analysis a)

Binomial logistic regression models were used to evaluate associations between genotype (allele count model) and wheeze phenotype

(outcome). Pairwise odds ratio (OR) with 95% confidence interval (95% CI) were reported separately for MTW vs VW vs NW, respectively. Significance was reported on nominal ($p \leq .05$) and SNP-adjusted levels ($p \leq .0071$).

Genotype-Gene Expression Association (analysis b)

To determine whether SNPs were cis- or trans-eQTLs for selected genes, a robust linear regression was performed. *Cis*-eQTLs refer to genetic variations acting on local genes (distance ≤ 1 Mb) and *trans*-eQTLs on distant genes (distance > 1 Mb or different chromosomes). Risk allele count for SNPs (additive allele count) was used as an independent variable and mRNA expression (ΔCT) as a dependent variable. Significance was reported on nominal ($p \leq .05$) and SNP and gene expression adjusted levels ($p \leq .0014$). Single SNP effects on gene expression were adjusted for PC1. Multi-collinearity between SNP and PC1 was assessed with the variance inflation factor (VIF). This allows classifying the SNP as (17q12-21 locus) independent when the adjusted effect was significant and VIF<5, and locus-dependent otherwise.

Gene Expression-Phenotype Association (analysis c)

Associations between gene expression and wheeze phenotype were modeled with robust binomial logistic regression, including the wheeze phenotype as a clinical outcome. The outcome was wheeze and the predictor variable mRNA expression (ΔCT). Significance was reported on nominal ($p \leq .05$) and gene expression adjusted levels ($p \leq .01$).

Mediation analyses (analysis d)

Causal mediation analyses were applied to quantify the effect of the genotype (for each SNP) on wheeze via gene expression. For this, a mediator model was specified as a linear model of risk allele on mRNA expression and an outcome model was used, which was a binomial logistic regression model of risk allele and mRNA expression on wheeze phenotype. The actual mediation analysis combined these two parts and estimated quantities such as "total effect", "indirect effect", "direct effect", and "proportion mediated" (Supporting-Information).

3 | RESULTS

3.1 | Study population

50.6% (91/180) of children developed wheeze until 6y. 14.4% (26/180) were classified as MTW and 36.1% (65/180) as VW (Table S3). Groups were significantly different regarding maternal education, any allergy, hay fever, and asthma. The effect estimates in analyses (a) and (c) were additionally adjusted for maternal education. No difference regarding birth season was observed ($p = .28$, not shown).

3.2 | Increased risk for multitrigger wheeze in 17q12-21 risk allele carriers (analysis a)

LD analyses were performed (Figure 1), leading to the selection of 12 reported SNPs in or close to eight genes within the 17q12-21 locus (Table S4). Genotyping quality, including allele frequencies and HWE showed no deviation between groups ($p > .05$). LD patterns in our population were highly comparable to ERP (Figure S3).

Risk allele frequency (RAF) for individual SNPs was highest in children with MTW, ranging from 46% to 86% (Table 1). The risk for MTW was particularly high in carriers of risk variants of rs9303277, rs12150079, rs2305480, rs7216389, and rs8079416 in comparison with NW (OR: 3.05–4.74) or VW (OR: 3.00–5.43) after adjustment for maternal education and multiple testing. All disease-related SNPs except rs8079416 were located in the core region, including *IKZF3*, *ZPBP2*, *GSDMB*, and *ORMDL3* genes, initially defined by Moffatt et al.[3,8] In contrast, no association for children with VW vs NW was detected. PC1 was used to estimate the overall effect of 23 genetic variants of the 17q12-21 locus on an individual's phenotype, showing significantly increased likelihood for MTW (vs both NW and VW; OR: 1.46). Furthermore, PC1 showed a very high correlation to a polygenic risk score (Figure S4).

In summary, risk allele carriers of 17q12-21 core region were at high risk to develop MTW but not VW (Figure S5).

3.3 | Risk variants within the 17q12-21 core region showed locus-dependent eQTL signals (analysis b)

Since novel eQTL analyses can capture gene regulatory processes after cell stimulation,[26] we assessed mRNA expression in unstimulated and LpA-/PHA stimulated CBMCs (stimulation effect, Figures S6-S7). All five core region risk variants and PC1 (significantly associated with MTW previously) were associated with increased *GSDMA*, *TLR2*, *TLR5*, and decreased *TGFB1* expression (Table 2, n = 35/71/73/44, respectively). After adjustment for multiple testing, *GSDMA* was significantly associated with PC1 and all five core region SNPs after PHA stimulation, while *TGFB1* was significantly associated with PC1 and rs2305480 after LpA stimulation. Additional associations were not significantly different (not shown).

To disentangle dependent and independent genetic signals of single SNPs on other 17q12-21 SNPs, the observed genotype-gene expression associations were adjusted for PC1. As result, almost all identified associations (Table 2) in the core region disappeared after

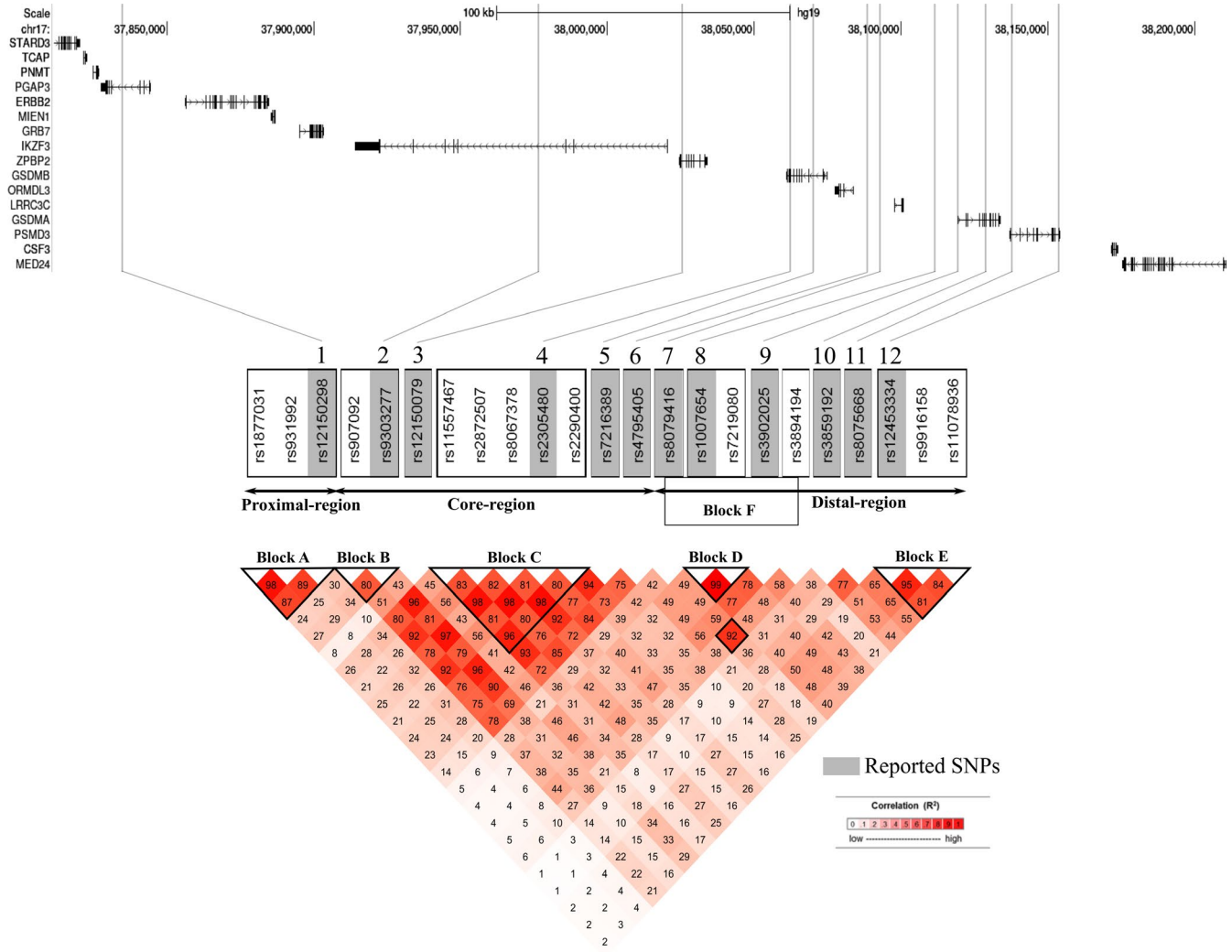

FIGURE 1 Pairwise linkage disequilibrium (LD) of assessed SNPs ($n = 23$). Overview of genes and risk variants present within the investigated 17q12-21 locus. Location of genes according to the human genome assembly GRCh37/hg19 using the UCSC Genome Browser. SNPs in high LD to each other ($r^2 > .8$, displayed in dark red) are shown in blocks (A-F). Selected representative SNPs ($n = 6$) from each of six blocks and single SNPs ($n = 6$, not connected to a block) have been assigned a consecutive number and reported in the results ($n = 12$, grey boxes)

the adjustment, meaning that the LD remains between these variants and the observed genotype-gene expression associations were not independent of each other, displaying mainly dependent eQTL effects (98.4%, Table 3A). Proximal and distal region SNPs showed relatively independent signals, 40% and 22.9%, respectively (Table 3B). After correction for multiple testing, three independent signals were identified for the distal region, rs1007654 with lower $CD209_{(U)}$, $TRAF6_{(PHA)}$, and $CD86_{(PHA),}$ rs3902025 with lower $RORA_{(LpA),}$ and rs12453334 with lower $IL\text{-}9_{(PHA)}$ expression (n = 47/62/41/50/157, respectively).

Taken together, risk variants within the core region showed locus-dependent effects, predominantly associated with *GSDMA* and *TGFB1*. SNPs within the distal region appeared to be more independent eQTL SNPs associated with *CD209*, *CD86*, *TRAF6*, *RORA*, and *IL-9* (Figure S5).

3.4 | Different mRNA-based immune signatures enable the identification of multitrigger and viral wheeze already at birth (analysis c)

Increased expression of innate genes in unstimulated CBMCs was observed in MTW for *ORMDL3*, *IPS1*, *NLRP3*, and *TLR2*, while decreased levels of *TNFAIP3* and *TNIP2* (also involved in NF-κB signaling), and *CD274* were associated with VW when compared to children with NW (Table 4, n = listed in the table). Additionally, increased levels of innate genes (*CASP1*, *IFIH1*, *IPS1*, *LY75*, *NLRP3*, *TLR2*) and *ORMDL3* were associated with a higher risk for subsequent MTW but not for VW. After adjustment for maternal education and multiple testing, increased *IPS1* and *LY75* expressions showed a significant association with subsequent MTW vs NW/VW, respectively, and decreased *TNFAIP3* and *TNIP2* expressions with VW vs NW (Figure S5).

TABLE 1 Associations of the overall 17q12-21 locus effect (PC1) and assessed risk variants with subsequent wheeze phenotype (analysis a)

	RAF [%]			MTW vs. NW			MTW vs. VW			VW vs. NW		
SNP	MTW	VW	NW	OR_{adj}	(95% CI)	*p*-value	OR_{adj}	(95% CI)	*p*-value	OR_{adj}	(95% CI)	*p*-value
PC1[1]	–	–	–	1.46	(1.17; 1.82)	**.001**†	1.46	(1.15; 1.86)	**.002**†	1.03	(0.91; 1.18)	.61
#1 rs12150298	46	28	29	2.48	(1.17; 5.27)	**.02**	3.90	(1.49; 10.17)	**.005**	0.93	(0.54; 1.60)	.78
#2 rs9303277	73	45	43	4.40	(1.92; 10.09)	**.0005**†	3.88	(1.68; 8.98)	**.002**†	1.09	(0.68; 1.74)	.72
#3 rs12150079	86	65	66	4.29	(1.60; 11.52)	**.004**†	5.43	(1.80; 16.39)	**.003**†	0.98	(0.58; 1.65)	.93
#4 rs2305480	73	50	49	3.05	(1.42; 6.55)	**.004**†	3.18	(1.40; 7.23)	**.006**†	1.06	(0.65; 1.72)	.81
#5 rs7216389	69	46	43	3.55	(1.64; 7.71)	**.001**†	3.23	(1.44; 7.27)	**.005**†	1.12	(0.70; 1.80)	.63
#6 rs4795405	71	54	49	2.74	(1.30; 5.77)	**.008**†	2.53	(1.10; 5.83)	**.03**	1.31	(0.79; 2.17)	.29
#7 rs8079416	68	45	39	4.74	(2.07; 10.86)	**.0002**†	3.00	(1.33; 6.75)	**.008**†	1.38	(0.83; 2.27)	.21
#8 rs1007654	75	62	62	1.84	(0.86; 3.93)	.12	1.91	(0.84; 4.33)	.12	1.09	(0.67; 1.77)	.74
#9 rs3902025	71	55	53	2.11	(1.05; 4.21)	**.04**	2.35	(1.03; 5.36)	**.04**	1.15	(0.72; 1.83)	.56
#10 rs3859192	62	44	42	2.01	(1.07; 3.80)	**.03**	2.05	(1.00; 4.20)	.05	1.16	(0.73; 1.84)	.53
#11 rs8075668	67	53	49	2.02	(1.00; 4.07)	.05	1.83	(0.87; 3.87)	.11	1.28	(0.79; 2.08)	.32
#12 rs12453334	73	61	61	1.64	(0.79; 3.40)	.19	1.88	(0.84; 4.18)	.12	1.08	(0.67; 1.74)	.75

The model was adjusted for maternal education ($_{adj}$), $p \leq .05$ (bold black) and for multiple testing, $p \leq .0071$ (†, bold black).

Abbreviations: *PC1*, first principal component of the 23 SNPs; *RAF*, risk allele frequency; *MTW*, multitrigger wheeze ($n = 26$), *CI*, confidence interval from logistic regression; *OR*, odds ratio; *SNP*, single nucleotide polymorphism (consecutive number and rs-ID); *VW*, viral wheeze ($n = 65$); *NW*, no wheeze ($n = 89$); -, not applicable.

[1]due to the scale of PC1, the effect size is not interpretable as for single SNPs.

TABLE 2 Main associations of 17q12-21 risk variants at the core region and the overall locus effect (PC1) with mRNA expression [change in ΔCT] (analysis b)

Genes	PC1[1]	#2 rs9303277	#3 rs12150079	#4 rs2305480	#5 rs7216389	#6 rs4795405
GSDMA	**0.46 PHA***(†)**	**1.49 PHA***(†)**	**1.95 PHA***(†)**	**1.61 PHA***(†)**	**1.51 PHA***(†)**	**1.75 PHA***(†)**
CD209	0.26 U	1.18 U*	1.32 U*	1.35 U**	0.89 U	1.31 U*
LY75	0.21 PHA*	0.83 PHA*	0.96 PHA*	0.74 PHA*	0.69 PHA*	0.53 PHA
MYD88	0.16 PHA	0.36 PHA	0.07 PHA	0.25 PHA	0.34 PHA	0.21 PHA
NLRP3	0.16 PHA*	0.73 PHA**	0.68 PHA*	0.58 PHA*	0.64 PHA*	0.55 PHA
TLR2	0.41 PHA**	1.35 PHA**	1.59 PHA**	1.47 PHA**	1.12 PHA*	1.25 PHA*
TLR4	-0.20 U	-0.22 U	-1.09 U*	-0.46 U	-0.38 U	-0.60 U
TLR5	0.32 PHA**	1.14 PHA**	1.58 PHA**	1.16 PHA**	1.18 PHA**	1.00 PHA*
TLR7	0.26 PHA*	0.85 PHA	1.15 PHA*	0.58 PHA	0.75 PHA	0.66 PHA
TNIP2	-0.21 U*	-0.48 U	-0.56 U	-0.48 U	-0.56 U	-0.54
TRAF6	-0.09 U 0.30 LpA* 0.01 PHA	0.03 U 1.07 LpA* 0.35 PHA	-0.36 U 0.60 LpA -0.06 PHA	-0.06 U 1.22 LpA* 0.26 PHA	-0.02 U 1.06 LpA 0.31 PHA	-0.19 U 1.21 LpA* 0.20 PHA
CD274	-0.31 U*	-0.71 U	-1.20 U	-0.80 U	-0.78	-0.98 U
CD86	0.16 LpA	1.67 LpA*	-1.00 LpA	0.60 LpA	1.53 LpA	0.57 LpA
GATA3	0.09 PHA	0.34 PHA*	0.28 PHA	0.29 PHA	0.27 PHA	0.27 PHA
IL-9	0.02 PHA	0.30 PHA	0.11 PHA	0.23 PHA	0.45 PHA	0.24 PHA
IL-10	-0.17 U	-0.53 U	-0.93 U*	-0.67 U*	-0.48 U	-0.53 U
IL-22	0.03 PHA	0.35 PHA	-0.04 PHA	0.24 PHA	0.43 PHA	0.17 PHA
IL-23R	-0.06 LpA	0.25 LpA	-0.21 LpA	-0.26 LpA	-0.04 LpA	-0.43 LpA
IRF1	-0.09 LpA	-0.16 LpA	-0.21 LpA	-0.25 LpA	-0.24 LpA	-0.47 LpA*
RORC3	0.01 LpA 0.19 PHA*	0.35 LpA 0.63 PHA*	0.30 LpA 0.33 PHA	0.05 LpA 0.45 PHA	0.13 LpA 0.60 PHA*	-0.17 LpA 0.52 PHA
TGFB1	**-0.65 LpA***(†)** -0.41 PHA*	-1.79 LpA* -1.27 PHA*	-2.32 LpA* -1.52 PHA*	**-2.22 LpA**(†)** -1.24 PHA*	-1.73 LpA* -1.52 PHA*	-2.21 LpA** -1.22 PHA

Main findings (nominal $p \leq .05$, black letters, grey otherwise) are shown with effects and p-values from linear regression, annotated with $p \leq .05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), and multiple test adjusted, $p \leq .0014$ (†, bold black). Numbers show mean changes in mRNA expression [ΔCT] due to the +1 risk allele. The numbers of children with highly significant findings (***, †) differ depending on the genotype and selected genes between $n = 34$-35.

Abbbreviations: PC1, first principal component; *LpA*, lipid A stimulated; *PHA*, phytohemagglutinin stimulated; *U*, unstimulated CBMCs.

[1]Due to the scale of PC1, the effect size is not interpretable as for single SNPs.

3.5 | 17q12-21 risk-variant effect on multitrigger wheeze was partially mediated by upregulated TLR2 expression (analysis d)

Finally, we conducted mediation analyses to assess whether the effect of carrying 17q12-21 risk variant on disease phenotypes was mediated by mRNA expression (indirect effect). As a result, the effect of PC1 and especially of rs9303277 (core region) on MTW was partially mediated by 34% to 36% through changes in $TLR2_{(PHA)}$ with an indirect effect OR: 1.03 (95% CI, 1.00–1.07, $p = .03$; Figure S8) and OR: 1.09 (95% CI, 1.01–1.22, $p = .03$; Figure 2A), respectively. In *trans*, rs9303277 regulates $TLR2_{(PHA)}$ expression (nominal $p = .006$) on chr.4 and in *cis* $GSDMA_{(PHA)}$ expression (nominal $p < .001$; Table 2, Figure 2B) that is approximately 143 kb distant from rs9303277. A correlation between both genes *GSDMA* and *TLR2* (r = .5, $p = .01$; Figure 2C) was observed, indicating that *cis*-modulation of *GSDMA* results in differential *TLR2* expression.

4 | DISCUSSION

In the PAULINA/PAULCHEN birth cohorts, our findings revealed the importance of genetic variants within the asthma-associated 17q12-21 locus, identifying core region SNPs associated with increased risk for MTW but not VW, partly mediated (34%–36%) by dysregulation of *TLR2* expression. Dependent effects of individual SNPs in the core region on other 17q12-21 SNPs on gene expression were observed (increased: *GSDMA*, *TLR2*, and *TLR5*; decreased: *TGFB1*), suggesting that pro-inflammatory priming and immunological imbalance already at birth may be critical for asthma pathophysiology. In

TABLE 3 Main findings of locus-independent effects of single SNPs on mRNA expression after adjustment for PC1 (A) at the core region and (B) at the proximal and distal region (analysis b)

(A)

Genes	#2 rs9303277	#3 rs12150079	#4 rs2305480	#5 rs7216389	#6 rs4795405
			core-region 1.6%[1]		
MYD88	-0.79	-2.3	-2.93	-1.04	-3.43
	PHA	PHA*	PHA	PHA	PHA
TLR4	1.56	-1.18	1.17	1.47	1.02
	U*	U	U	U	U
TRAF6	1.38	-0.21	1.36	1.52	0.97
	U*	U	U	U	U
	0.55	-0.97	0.83	0.25	0.75
	LpA	LpA	LpA	LpA	LpA
	1.58	-0.21	1.58	1.45	1.19
	PHA*	PHA	PHA	PHA	PHA
CD86	2.91	-3.11	-0.32	3.00	-0.48
	LpA*	LpA**	LpA	LpA*	LpA
IL-9	1.02	0.14	1.00	1.69	0.96
	PHA	PHA	PHA	PHA**	PHA
IL-22	1.24	-0.39	0.93	1.56	0.43
	PHA*	PHA	PHA	PHA*	PHA
IL-23R	2.11	0.00	-0.23	0.93	-1.14
	LpA*	LpA	LpA	LpA	LpA
RORC3	1.39	0.68	0.13	0.51	-1.19
	LpA*	LpA	LpA	LpA	LpA
	0.14	-0.78	-1.22	-0.16	-0.73
	PHA	PHA	PHA	PHA	PHA

(B)

Genes	#1 rs12150298	#7 rs8079416	#8 rs1007654	#9 rs3902025	#10 rs3859192	#11 rs8075668	#12 rs12453334
	proximal 40%[1]	distal-region 22.9%[1]					
GSDMA	-1.53 U**	1.22 U	2.46 U	0.99 U	0.19 U	0.77 U	1.21 U
	-0.31 PHA	0.61 PHA	0.49 PHA	1.53 PHA*	0.87 PHA	0.15 PHA	1.11 PHA
GSDMB	-1.18 LpA*	-0.98 LpA	-0.15 LpA	-1.07 LpA	-0.69 LpA	0.14 LpA	-0.03 LpA
CD209	1.33 U	-1.65 U	**-3.15 U**(†)**	-1.23 U	-0.26 U	-0.76 U	-0.99 U
	-0.32 LpA	1.50 LpA*	0.29 LpA	1.57 LpA*	0.94 LpA	0.08 LpA	0.1 LpA
IPS1	0.73 PHA**	-013 PHA	-0.34 PHA	-0.67 PHA*	-0.32 PHA	0.11 PHA	-0.04 PHA
NLRP3	0.12 U	0.58 U	-1.25 U*	0.07 U	0.34 U	-0.67 U	-0.65 U
TLR4	0.1 U	-0.25 U	-1.42 U*	-0.69 U	-0.59 U	-0.79 U	-0.64 U
	1.18 PHA*	-1.26 PHA	-1.59 PHA*	-1.27 PHA*	-0.99 PHA	-0.41 PHA	-0.12 PHA
TLR5	0.48 LpA	-0.3 LpA	-0.51 LpA	-0.35 LpA	0.79 LpA	1.28 LpA*	0.84 LpA
TLR7	-0.18 U	-0.14 U	0.12 U	1.78 U	1.57 U*	0.62 U	0.36 U
TNIP2	-0.98 U*	0.27 U	-0.33 U	0.06 U	0.24 U	0.08 U	-0.37 U
TRAF6	0.26 PHA	-0.25 PHA	**-2.04 PHA***(†)**	-0.82 PHA	-0.27 PHA	-0.89 PHA	-0.99 PHA*
CD274	-0.15 PHA	-0.12 PHA	-0.97 PHA	-0.47 PHA	-1.06 PHA	-1.63 PHA*	-0.83 PHA
CD80	-1.40 U*	1.79 U**	-0.87 U	-0.4 U	0.86 U	0.17 U	-1.13 U
	0.54 PHA	-0.12 PHA	-2.21 PHA**	-1.70 PHA*	-1.22 PHA*	-1.93 PHA**	-1.19 PHA
CD86	0.86 LpA	2.45 LpA	-2.29 LpA*	-1.23 LpA	-1.26 LpA	-1.45 LpA	-1.72 LpA
	1.28 PHA	-1.77 PHA	**-3.57 PHA*** (†)**	-2.59 PHA**	-2.09 PHA**	-2.54 PHA**	-1.71 PHA*
FOXP3	0.17 U	-0.28 U	-1.07 U*	-0.18 U	0.42 U	0.04 U	-0.16 U
IL17	-0.42 U	0.34 U	0.78 U*	0.74 U*	-0.14 U	-0.17 U	0.14 U
IL9	-0.34 PHA	0.12 PHA	0.35 PHA	0.63 PHA	-0.62 PHA	-1.11 PHA**	**-1.24 PHA**(†)**
IL22	0.00 PHA	-0.12 PHA	-0.26 PHA	0.16 PHA	-0.48 PHA	-0.85 PHA*	-1.13 PHA**
RORA	0.75 LpA	-0.50 LpA	-1.51 LpA**	**-1.88 LpA***(†)**	0.00 LpA	0.06 LpA	-0.64 LpA
	0.44 PHA	-0.17 PHA	-1.12 PHA*	-1.26 PHA*	0.25 PHA	0.52 PHA	-0.12 PHA
RORC4	0.34 LpA	-0.51 LpA	-1.2 LpA	-1.78 LpA*	0.09 LpA	0.12 LpA	0.21 LpA
STAT6	0.13 PHA	-0.07 PHA	-0.17 PHA	-0.50 PHA	0.21 PHA	0.57 PHA*	0.35 PHA
TGFB1	0.07 LpA	0.42 LpA	0.05 LpA	0.45 LpA	-0.80 LpA	-2.47 LpA*	-1.63 LpA

Main findings for independent eQTL effects (nominal $p \leq 0.05$ and VIF<5, black letters, grey otherwise) are shown for risk variants. Table 3A at the core region and Table 3B at the proximal and distal regions after adjustment for PC1. *p*-value is annotated with $p \leq .05$ (*), $p \leq 0.01$ (**), $p \leq .001$ (***) and multiple test adjusted $p \leq .0014$ (†, bold black). Numbers show mean changes in mRNA expression [ΔCT] due to the +1 risk allele. The numbers of children with highly significant findings (***, †) differ depending on the genotype and selected genes between n = 41-62-157.

Abbbreviations: *LpA*, lipid A stimulated; *PHA*, phytohemagglutinin stimulated; *U*, unstimulated CBMCs.

[1]The percentage of proximal/core/distal region refers to the number of observed associations after adjustment for PC1 (nominal $p \leq .05$) divided by all associations before the adjustment for PC1 for the same observed association (same stimulation/gene/SNP) for each region.

TABLE 4 Main findings of relevant associations between mRNA expression and subsequent wheeze phenotypes (analysis c)

		MTW vs NW			MTW vs VW			VW vs NW		
Gene	Stim	OR_{adj}	(95% CI)	*p*-value	OR_{adj}	(95% CI)	*p*-value	OR_{adj}	(95% CI)	*p*-value
ORMDL3	U	2.44	(1.02; 5.79)	**.045**	4.48	(1.25; 16.03)	**.02**	0.89	(0.47; 1.66)	.70
CASP1	U	2.16	(0.93; 5.04)	.08	1.62	(1.03; 2.55)	**.04**	0.96	(0.82; 1.12)	.59
IFIH1	U	1.57	(0.99; 2.49)	.06	1.38	(1.02; 1.85)	**.03**	0.90	(0.75; 1.09)	.29
IPS1	U	3.39	(1.35; 8.51)	**.01**†	2.59‡	(1.08; 6.17)‡	**.03**‡	1.06	(0.23; 4.91)	.94
LY75	U	2.34	(0.69; 7.97)	.17	2.92	(1.29; 6.59)	**.01**†	0.97	(0.76; 1.24)	.83
NLRP3	U	2.46	(1.19; 5.09)	**.02**	2.18	(1.15; 4.14)	**.02**	0.84	(0.63; 1.11)	.21
TLR2	PHA	1.75	(1.11; 2.75)	**.02**	1.54	(1.09; 2.17)	**.01**	1.06	(0.87; 1.30)	.53
TNFAIP3	U	0.93	(0.64; 1.37)	.74	1.39	(0.80; 2.41)	.24	0.45	(0.25; 0.81)	**.01**†
TNIP2	U	0.62	(0.19; 2.02)	.43	1.16	(0.73; 1.84)	.53	0.51	(0.32; 0.80)	**.003**†
CD274	U	0.78	(0.53; 1.14)	.21	1.11	(0.77; 1.58)	.58	0.70	(0.50; 0.99)	**.046**

The main findings are shown. Associations (OR (95% CI), *p*-values) adjusted ($_{adj}$) for maternal education, $p \leq .05$ (bold black) and multiple testing, $p \leq .01$ (†, bold black). The numbers of children with significant findings differ depending on the phenotype and selected genes between MTW/VW/NW n = 12-14/12-33/20-22. Odds ratio (OR) and 95% confidence interval (CI) were derived from a logistic regression model. ‡, unadjusted OR shown due to convergence issues.

Abbbreviations: *MTW*, multitrigger wheeze; *NW*, no wheeze; *PHA*, phytohemagglutinin stimulated CBMCs; *Stim*, stimulation; *U*, unstimulated CBMCs; *VW*, viral wheeze.

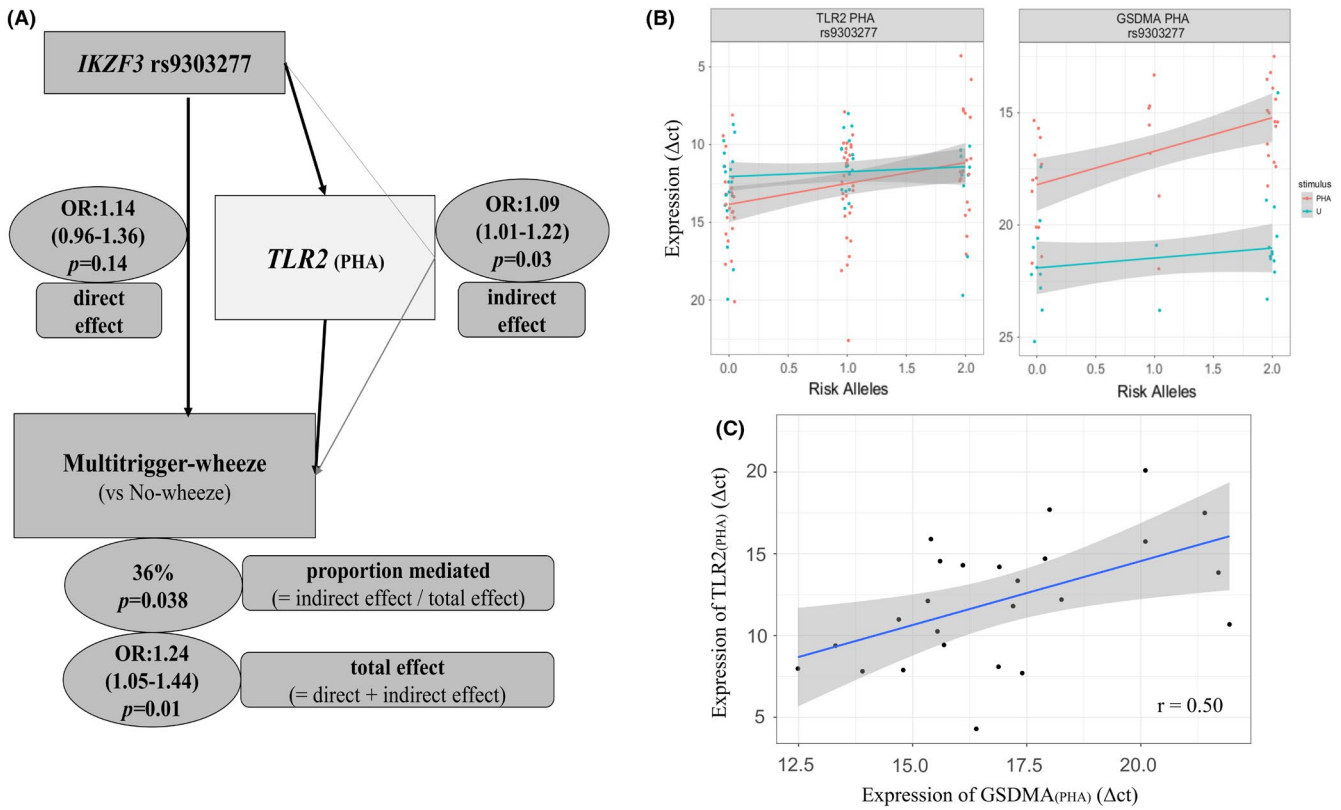

FIGURE 2 Effect of risk-variant rs9303277 on the risk of developing multitrigger wheeze (vs no wheeze) by the age of six mediated by *TLR2* expression in CBMCs (analysis d). (A) The effect of risk-variant rs9303277 on subsequent MTW vs NW (n = 34) is divided into a direct and an indirect effect by mediation analysis. $TLR2_{(PHA)}$ expression is shown as a partial mediator (36%) between risk variants in the 17q12-21 locus and subsequent MTW. (B) The allele-specific expression of *TLR2* (ΔCt, unstimulated n = 46) is induced upon PHA stimulation (n = 71) and enhanced in rs9303277 risk allele carriers (*trans*-regulation). *GSDMA* expression (ΔCt, unstimulated n = 24) was induced upon PHA stimulation (n = 35) and also enhanced in rs9303277 risk allele carriers (*cis*-regulation). (C) Spearman rank correlation of *GSDMA* and *TLR2* expression upon PHA stimulation (n = 25, r = .50, p = .01)

contrast, independent effects were observed in the distal region, particularly for rs1007654 on *CD209*, *TRAF6*, and *CD86* expression, suggesting that this SNP may be involved in critical processes required for maturation/activation/development of dendritic cells (DCs) and initiation of DC-T-cell interaction. Identification of these novel immunoregulatory mechanisms is critical.

4.1 | Increased risk for multitrigger wheeze in 17q12-21 risk allele carriers (analysis a)

Being aware of the rapidly developing field on childhood asthma genetics and additional loci within and beyond chr17, we focused on a contribution of still unknown underlying mechanisms of 17q12-21 on childhood asthma. In previous studies, 17q12-21 SNPs were associated with wheeze.[6,7,24,27] Identifying that children with 17q12-21 core region SNPs are more likely to develop MTW adds another facet and is important for several reasons. MTW, but not VW, has been repetitively suggested to correlate with a higher susceptibility for subsequent asthma.[28] Thus, symptoms of MTW may represent the first clinical indication of asthma together with an underlying genetic risk. Notably, in our birth cohort, VW was defined as parent-reported wheeze during (viral) infection. As viral infection can subsequently lead to bacterial infections, this may in part contribute to differences in findings with VW caused by, for example, rhinovirus only, observed in other studies.[29,30] Besides defining wheeze by symptoms, temporal patterns are important. Yet, a symptom-based approach has direct implications on current clinical decisions, as sequential phenotyping requires at least 1–2 years of follow-up. We acknowledge that there is a heterogeneity of wheeze phenotypes and a group of children with symptoms will be transitioning from VW to MTW, and a small group *vice versa*. However, a larger percentage of children with VW can be distinguished clinically and grouped as a sole entity. A family history of asthma is associated with childhood asthma. However, in our cohort, the effect on MTW/VW was not large enough to reach statistical significance. Larger studies might quantify the contribution of family anamnesis on the functional regulation in newborns for the effects we observed between wheeze phenotypes.

4.2 | Risk variants within the 17q12-21 core region showed locus-dependent eQTL signals (analysis b)

Although many of these 17q12-21 SNPs are associated with wheeze/asthma, it is still unclear through which downstream mechanisms these risk variants cause disease development. Previously, we demonstrated *cis*-regulatory effects in risk allele carriers with increased *ORMDL3* and *GSDMA* expression at birth.[10] In the present study, we extended the findings that 17q12-21 risk variants may have a *trans*-regulatory potential, mainly for immune regulatory genes. A strong genetic association signal in all five core region SNPs was detected for *GSDMA*, *TLR2*, *TLR5*, and *TGFB1* expression, which may point to pro-inflammatory priming in asymptomatic children already at birth. *TLR2* and *TLR5* activation drives pro-inflammatory NF-κB regulation, *GSDMA* upregulation initiated pyroptosis, cell death, and strong inflammation.[31] Concomitantly, *TGFB1* downregulation may lead to a regulatory immune imbalance[32] at birth related to the pathophysiology of wheeze. Notably, core region SNPs rs7216389 and rs2305480 were associated with *GSDMA* expression in a recent study,[33] the latter also being the leading SNP for early-life wheeze[7] and childhood-onset asthma.[9] Furthermore, another study showed an association between *GSDMB* and *TGFB1* expression.[34] In our study, rs2305480, a missense variant located in *GSDMB*, was associated with MTW and *TGFB1* downregulation, confirming the connection and providing further evidence for possible mechanistic pathways. Furthermore, most independent eQTL SNPs at the distal region showed no significant association with MTW nor VW but decreased expression of *CD209*, *CD86*, *TRAF6*, *RORA*, and *IL-9*. *TRAF6* is involved in the regulation of critical processes required for DC maturation.[35] *CD209* and *CD86* expressed on DCs play a pivotal role in DC-T-cell communication.[36,37] All three genes were associated with risk-variant rs1007654 located within a regulatory region nearby *GSDMA*, associated with early-onset asthma.[38] The connection to DCs provides a new component requiring further functional investigation to determine a potential role of this risk variant in DC maturation/activation.

We are aware of other possibilities to identify independent genetic signals, for example, adjusting for the most significant SNP. However, adjusting for PC1 that summarizes the overall genetic signal within 17q12-21 locus (explaining 60% of the variance) is less arbitrary and phenotype-independent, resulting in better robustness and comparability of different genotype-gene expression associations. Additionally, PC1 showed a correlation ($r > .99$) to a polygenic risk score built of the 23 SNPs using the coefficients reported by Moffatt et al.[3] Thus, PC1 is directly interpretable as a polygenic risk score. Furthermore, controlling the false-positive error rate by Bonferroni correction when exploring highly correlated genetic associations with limited sample size results in an overadjustment with excessive false negatives, masking findings we find worth reporting.[39] We, therefore, applied a less stringent alternative and estimated the effective dimensions by using PCA for SNP data (7 dimensions) and hierarchical clustering for gene expression data (5 dimensions) and adjusted the results by those.

To date, little evidence for *trans*-eQTL SNPs and downstream biological regulation exists in mononuclear cells related to the progression of asthma. Nevertheless, our findings suggest that 17q12-21 variants contribute to mechanisms regulating immune responses already at birth, influencing early immune priming during maturation, which may contribute to asthma susceptibility. Furthermore, our study indicates different effects of 17q12-21 regions (proximal/core/distal), supporting the hypothesis that core region SNPs may be more relevant for early-life susceptibility to childhood-onset asthma, confirming the novel findings by Hallmark et al.[7] proposing chr17q12-21 as a "wheezing-locus."

4.3 | Different mRNA-based immune signatures enable the identification of multitrigger and viral wheeze already at birth (analysis c)

The findings of distinct mRNA expression at birth associated with MTW support the role of early priming of specific maturational facets. For *TLR2*, strong PHA stimulation was necessary, indicating a requirement of specific induction of innate activation of regulatory components early in life. Importantly, different mRNA-based immune signatures were detected, specific for either MTW (*IPS1* and *LY75*) or VW (*TNFAIP3* and *TNIP2*). These findings can help distinguish NW and VW from MTW, who were rather prone to develop asthma in our cohort. Our effects were identified in CBMCs; thus, tissue-specific associations and investigation of involved pathways during disease development require further studies. The focus on cord blood mRNA expression to elucidate the importance of early-life immunity on wheeze and the longitudinal follow-up in these children are strengths of this study. However, due to the low sample size in subgroups, no further stratification was feasible. Future larger studies and in-depth functional assessments are required to further disentangle the exact mechanism of "asthma-promoting immune regulation."

4.4 | 17q12-21 risk-variant effect on multitrigger wheeze was partially mediated by upregulated TLR2 expression (analysis d)

Finally, the risk for MTW in 17q12-21 risk variant carriers via *TLR2* was identified for overall locus effect and especially for the core region SNP rs9303277 (*IKZF3* intron 3) using the mediation analysis. So far, no functional prediction is known for rs9303277. The cell-specific *cis*- and *trans*-regulatory potential of these non-coding SNPs has not been fully characterized. Our *trans*-eQTL analyses suggest that rs9303277 regulates the expression of *TLR2* via *GSDMA* as a "*cis*-mediator transcript" only visible after PHA stimulation, a yet unknown relationship. Increased *TLR2* expression in MTW was linked to the initiation of inflammation and promoting Th2-mediated regulation, potentially contributing to asthma pathogenesis. Our previous studies indicate that the TLR pathway and *TLR2* variants influence T-regulatory cell function in CBMCs, which were in turn closely linked to asthma development.[16,40] This current study indicates that 17q12-21 risk variants and innate immunity receptors indirectly impact the risk of childhood-onset asthma. While this finding represents one potential mechanism of many with an attributable mediation of 36%, other heterogenetic mechanisms including epigenetics and environmental exposures are conceivable and require further assessment. Overall, our results may indicate interconnected regulations between *trans*-eQTL and *cis*-mediator transcripts providing novel insights about downstream pathways for asthma pathogenesis.

In conclusion, our findings suggest that in-depth analyses of gene regulation at birth together with chr17q12-21 genotyping are valuable for understanding the initiation of childhood wheeze and possibly asthma development. We disentangled that risk allele carriers of 17q12-21 core region showed an inflammatory immune pattern (*TLR2* increase) already at birth, both leading to an increased risk for MTW until the age of six. While this study focused on mRNA expression in CBMCs and showed potential relation between the mechanisms of asthma-related genetic variants and immune responses at birth, functional studies on hereby-identified *trans*-eQTLs will be essential to gain further insights into novel mechanistic immunological pathways and key regulatory mechanisms. These results show one of several possible mechanisms involved in the development of asthma and may pave the ground for future strategies for more precise prediction of disease development or represent drug targets with a greater probability of clinical success.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest in relation to this study.

AUTHOR CONTRIBUTIONS

Kristina Laubhahn: Data curation (lead); Formal analysis (supporting); Investigation (lead); Visualization (supporting); Writing – original draft (lead); Writing – review & editing (supporting). **Andreas Böck:** Data curation (supporting); Formal analysis (lead); Methodology (supporting); Visualization (supporting); Writing – review & editing (supporting). **Kathrin Zeber:** Data curation (supporting). **Sandra Unterschemmann:** Formal analysis (supporting). **Sonja Kunze:** Methodology (supporting). **Michaela Schedel:** Conceptualization (supporting); Methodology (supporting); Writing – review & editing (supporting). **Bianca Schaub:** Conceptualization (lead); Funding acquisition (lead); Project administration (lead); Supervision (lead); Writing – review & editing (lead).

ETHICS APPROVAL AND TRIAL REGISTRATION STATEMENT

All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the human ethics committee (Bavarian Ethical Board; LMU Munich #04092). Written informed consent for participation and publication was obtained from parents.

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