Genetic regulation of *IL1RL1* methylation and IL1RL1-a protein levels in asthma

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***Interleukin-1 receptor–like 1* (*IL1RL1*) SNPs regulate *IL1RL1*-methylation and serum IL1RL1-a levels, yet these effects are not related to asthma** http://ow.ly/AStC30hSvGy

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ABSTRACT *Interleukin-1 receptor–like 1* (*IL1RL1*) is an important asthma gene. (Epi)genetic regulation of *IL1RL1* protein expression has not been established. We assessed the association between *IL1RL1* single nucleotide polymorphisms (SNPs), *IL1RL1* methylation and serum IL1RL1-a protein levels, and aimed to identify causal pathways in asthma.

Associations of *IL1RL1* SNPs with asthma were determined in the Dutch Asthma Genome-wide Association Study cohort and three European birth cohorts, BAMSE (Children/Barn, Allergy, Milieu, Stockholm, an Epidemiological survey), INMA (Infancia y Medio Ambiente) and PIAMA (Prevention and Incidence of Asthma and Mite Allergy), participating in the Mechanisms of the Development of Allergy study. We performed blood DNA *IL1RL1* methylation quantitative trait locus (QTL) analysis (n=496) and (epi)genome-wide protein QTL analysis on serum IL1RL1-a levels (n=1462). We investigated the association of *IL1RL1* CpG methylation with asthma (n=632) and IL1RL1-a levels (n=548), with subsequent causal inference testing. Finally, we determined the association of IL1RL1-a levels with asthma and its clinical characteristics (n=1101).

IL1RL1 asthma-risk SNPs strongly associated with *IL1RL1* methylation (rs1420101; $p=3.7\times10^{-16}$) and serum IL1RL1-a levels ($p=2.8\times10^{-56}$). *IL1RL1* methylation was not associated with asthma or IL1RL1-a levels. IL1RL1-a levels negatively correlated with blood eosinophil counts, whereas there was no association between IL1RL1-a levels and asthma.

In conclusion, asthma-associated *IL1RL1* SNPs strongly regulate *IL1RL1* methylation and serum IL1RL1-a levels, yet neither these *IL1RL1*-methylation CpG sites nor IL1RL1-a levels are associated with asthma.

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Introduction

The heritability of asthma has been estimated to be around 60% [1] and large-scale genome-wide association studies (GWAS) have identified multiple susceptibility loci [2, 3]. One gene consistently found in asthma GWAS is *interleukin-1 receptor–like 1* (*IL1RL1*), which encodes a member of the Toll-like/IL-1 receptor superfamily expressed on inflammatory and resident cells in the lung [4–7]. Single nucleotide polymorphisms (SNPs) in *IL1RL1* have been associated with (time to onset of) asthma and atopic traits [2, 3, 8–16]. *IL1RL1* encodes three protein isoforms: IL1RL1-a (soluble ST2), which can be measured in serum; a transmembrane receptor, IL1RL1-b (S2TL); and two less well-characterised isoforms, isoform 3 and IL1RL1-c (ST2V) [17]. IL1RL1-a, IL1RL1-b and IL1RL1-c are all expressed in the lung [18, 19]. Binding of IL-33 to a heterodimeric receptor complex composed of IL1RL1-b and IL1RAcP on Th2 cells, innate immune cells (*e.g.* basophils and mast cells) and Type 2 innate lymphoid cells activates an MYD88-mediated inflammatory signalling cascade, contributing to airway inflammation by releasing pro-inflammatory Th2 cytokines such as IL-4, IL-5 and IL-13 [20]. IL1RL1-a is thought to serve as a decoy receptor, sequestering IL-33 and inhibiting its function [21–23].

The precise role of SNPs and methylation of *IL1RL1* in regulating the expression of IL1RL1 remains poorly understood. SNPs in *IL1RL1* are associated with DNA methylation at 5′-C-phosphate-G-3′ (CpG)

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sites (methylation quantitative trait loci (meQTL)) and affect protein levels (protein quantitative trait loci (pQTL)) and function [24]. SNPs in *IL1RL1* have previously been found to relate to IL1RL1-a levels in serum and bronchoalveolar lavage (BAL) fluid [25, 26]. However, it is unknown if *IL1RL1* SNPs are associated with methylation or how this relates to IL1RL1 protein expression and asthma development.

In this study, we analysed the relation between *IL1RL1* SNPs, *IL1RL1* gene methylation and serum IL1RL1-a protein levels. By integrating these multiple layers of data we aimed to reveal the genomic mechanism of *IL1RL1* in asthma.

Methods

A detailed description of the Methods is provided in the supplementary material.

Study populations

For phenotypic and genetic analyses, we used samples from the Dutch Asthma GWAS (DAG) cohort (n=1885) [27] and three different European birth cohorts that contributed to the MeDALL (Mechanisms of the Development of Allergy) project [28]: PIAMA (Prevention and Incidence of Asthma and Mite Allergy) (n=1913) [29], BAMSE (Children/Barn, Allergy, Milieu, Stockholm, an Epidemiological survey) (n=385) [30] and INMA (Infancia y Medio Ambiente) (n=320) [31]. Epigenetic analyses were performed in the three MeDALL cohorts (n=632). All studies were approved by medical ethics committees, and informed (parental) consent was obtained from all participants.

Asthma diagnosis

In the DAG cohort, asthma was defined as a doctor's diagnosis of asthma, asthma symptoms and the presence of airway hyperresponsiveness (AHR). In controls, neither asthma nor AHR was present. In the PIAMA, BAMSE and INMA cohorts, asthma was based on the published classical asthma definition of MeDALL [32], in which two of the following three criteria had to be positive: 1) doctor diagnosis of asthma ever, 2) use of asthma medication during the past 12 months and 3) wheezing/breathing difficulties in the past 12 months.

Selection of IL1RL1 *region SNPs*

For candidate gene analyses, we defined the *IL1RL1* region as all *IL1RL1* exonic and intronic sequences, as well as the juxtaposed genomic regions 200 kb 5′ to the transcription start site and 200 kb 3′ to the last exon. We verified linkage disequilibrium (LD) patterns of SNPs with a minor allele frequency (MAF) >0.01 in this region using data from the 1000 Genomes CEU panel (version 3, March 2012) [33].

(Epi)genetic data and serum IL1RL1-a levels

Details on genotyping and imputation are provided in the supplementary material. In the MeDALL study, *IL1RL1* DNA methylation of whole blood DNA collected at the age of 4 years was measured using Illumina 450k Methylation Beadchips (Illumina Inc., San Diego, CA, USA).

Serum IL1RL1-a protein levels in the DAG, BAMSE and INMA cohorts were measured using ST2/IL-1 R4 Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), and have previously been reported in PIAMA using an ST2 ELISA kit (Medical & Biological Laboratories Co., Woburn, MA, USA) [25].

Statistical analysis

We examined the associations of *IL1RL1* gene variants with asthma and of IL1RL1-a expression with asthma-related traits in the DAG and MeDALL cohorts. We performed genome-wide SNP and epigenome-wide CpG association analyses of serum IL1RL1-a levels in DAG and PIAMA, with replication in BAMSE and INMA. To assess if the SNPs in different LD blocks had independent effects on IL1RL1-a serum levels, we performed conditional analysis in the DAG cohort using SPSS 22.0 (IBN, Armonk, NY, USA). We performed candidate *IL1RL1* CpG meta-analysis on asthma and IL1RL1-a levels in the MeDALL cohorts. Causal inference testing was performed in PIAMA, the cohort with the largest sample size of complete data (SNPs, methylation, protein) (for details, refer to the supplementary material). We defined an (epi)genetic association as being significant when the p-value was below the Bonferroni-corrected threshold.

Results

Study populations

Clinical characteristics of the participants of the DAG and MeDALL cohorts are summarised in supplementary tables S1 and S2a–c. The DAG cohort comprised mostly adults with moderate to more severe asthma and spouse controls, whereas the PIAMA, BAMSE and INMA birth cohorts assessed

TABLE 1 Overview performed studies

Analysis	Association	Tissue	DAG	PIAMA	BAMSE	INMA	Meta-analysis[#]
Asthma association	*IL1RL1* SNPs - Asthma (% cases)		1885 (48.2)	**1913 (9.8)**	**385 (28.6)**	**322 (18.3)**	2620 (13.8)
	Age years		36.0±16.4	4.1±0.2	4.3±0.2	4.4±0.2	
Methylation QTL	*IL1RL1* SNPs - *IL1RL1* CpG islands	Blood		**226**	**88**	**182**	496
	Age years			4.1±0.2	4.3±0.2	4.4±0.2	
Protein QTL	Genome-wide SNPs - IL1RL1-a	Serum	564	595			
	IL1RL1 SNPs - IL1RL1-a	Serum	**564**	**595**	**83**	**220**	1462
	Age years		40.4±13.3	6.3±2.3	4.2±0.2	4.4±0.2	
Candidate CpG	*IL1RL1* methylation - Asthma (% cases)	Blood		**231 (15.2)**	**201 (22.3)**	**200 (17)**	632 (18.0)
	Age years			4.1±0.2	4.3±0.2	4.4±0.2	
EWAS	Genome-wide methylation - IL1RL1-a	Blood		**122**	**228**	**198**	548
	Age years			4.1±0.2	4.2±0.2	4.4±0.2	
Phenotype	IL1RL1-a - Asthma (% cases)	Serum	573 (81.8)	**632 (12.4)**	**184 (15.7)**	**285 (22.8)**	1101 (14.6)
	Age years		40.4±13.3	6.2±2.3	4.3±0.2	4.4±0.2	

Data are presented as n, where n is the number of subjects with available data in the cohort. Age is presented as mean±SD. DAG: Dutch Asthma Genome-wide Association Study; PIAMA: Prevention and Incidence of Asthma and Mite Allergy; BAMSE: Children/Barn, Allergy, Milieu, Stockholm, an Epidemiological survey; INMA: Infancia y Medio Ambiente; IL1RL1: interleukin-1 receptor-like 1; SNP: single nucleotide polymorphism; QTL: quantitative trait locus; CpG: 5′-C-phosphate-G-3′; EWAS: epigenome-wide association study. [#]: Cohort numbers in bold were included in the meta-analysis.

children with milder asthma and controls from the general population. An overview of all analyses is provided in table 1.

IL1RL1 *genomic region*

The genomic region spanning 200 kb up- and downstream from the *IL1RL1* gene (GRCh37/hg19; chr2:102,728,004–103,168,041) encompasses the *IL1R1*, *IL1RL2*, *IL18R1*, *IL18RAP* and *SLC9A4* genes. In total, 2229 overlapping SNPs were available in all cohorts. A highly complex LD pattern was identified, with LD blocks extending into neighbouring genes (r^2>0.7; 33 LD blocks) (supplementary figure S1). An overview of *IL1RL1* and its transcripts is provided in figure 1.

Association of IL1RL1 *SNPs with asthma*

In the DAG cohort, nominally significant associations were found between *IL1RL1* SNPs and asthma (*e.g.* rs6543119, beta=0.14, p=0.03). In the PIAMA cohort and in the MeDALL meta-analysis, nominally significant results were also found between asthma at 4 years of age and SNPs located in genes next to *IL1RL1* (*e.g.* rs7572871 (*IL1R2*): PIAMA beta=0.35, p=0.008; MeDALL meta-analysis beta=0.27, p=0.01) (table 2).

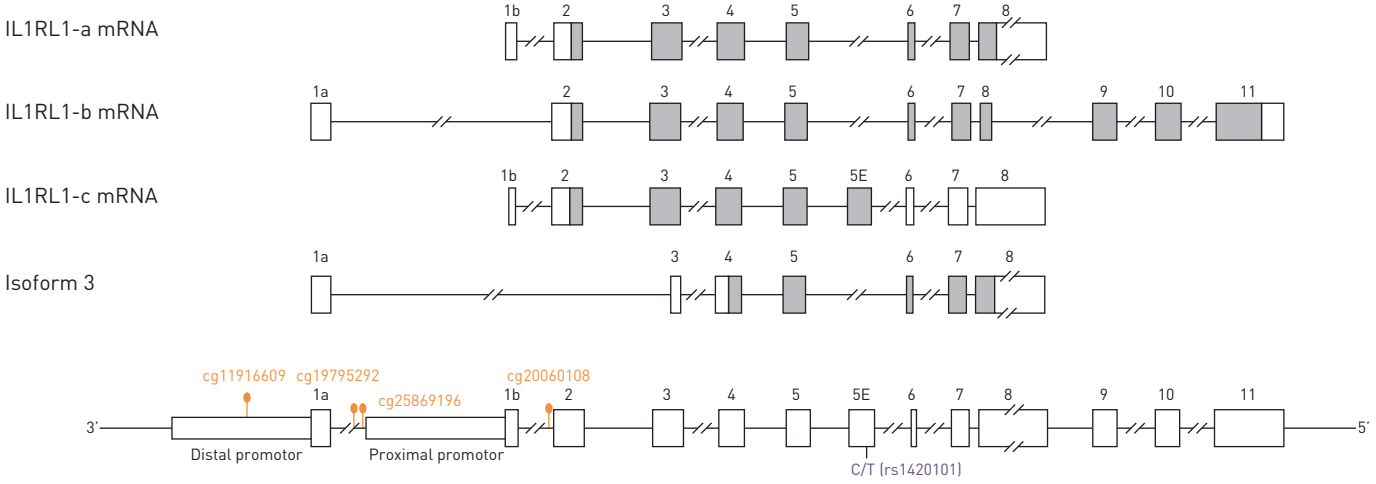

FIGURE 1 The *interleukin-1 receptor-like 1* (*IL1RL1*) gene (GRCh37/hg19; chr2:102,927,962–102,968,497) with transcript annotation of IL1RL1-a (ENST00000311734.6), IL1RL1-b (ENST00000233954.5), IL1RL1-c (ENST00000427077.1) and isoform 3 (ENST00000404917.6). The locations of studied 5′-C-phosphate-G-3′ sites (cg11916609, cg19795292, cg25869196, cg20060108) and the *IL1RL1* methylation and protein-associated single nucleotide polymorphism rs1420101 are presented. Exons are numbered. Grey regions are the transcribed parts of the exons.

TABLE 2 Results of the asthma, *interleukin-1 receptor-like 1* (*IL1RL1*) methylation and IL1RL1-a protein analyses from *IL1RL1* region SNPs selected from the five LD blocks most strongly associated with gene methylation and IL1RL1-a levels

			Asthma				Whole blood DNA methylation								IL1RL1-a levels	
			DAG		MeDALL[+]		cg11916609		cg19795292		cg25869196		cg20060108		Meta-analysis[§]	
SNP	A2[#]	LD[¶]	Beta	p-value	Beta	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value	Z	p-value
rs1420101[f]	T	1	0.10	1.27E−01	0.06	4.50E−01	−4.97	**6.88E−07**	−8.55	**1.20E−17**	−8.15	**3.73E−16**	−5.45	**5.18E−08**	−15.81	**2.83E−56**
rs11685424	A	2	0.04	5.03E−01	0.01	9.28E−01	−4.67	**3.05E−06**	−6.72	**1.81E−11**	−9.17	**4.56E−20**	−0.57	5.67E−01	−13.99	**1.88E−44**
rs13015714	T	3	0.05	5.46E−01	−0.07	5.00E−01	−4.66	**3.17E−06**	−1.75	8.01E−02	−4.29	**1.82E−05**	0.72	4.72E−01	−9.53	**1.53E−21**
rs1035130[f]	T	4	0.08	2.57E−01	0.14	1.20E−01	−3.29	1.02E−03	−8.37	**5.77E−17**	−6.94	**3.85E−12**	−5.81	**6.37E−09**	−10.76	**5.53E−27**
rs10192157[§]	T	5	−0.11	1.02E−01	−0.10	2.35E−01	0.61	5.40E−01	6.18	**6.49E−10**	3.71	2.05E−04	5.66	**1.52E−08**	6.62	**3.57E−11**

DAG: Dutch Asthma Genome-wide Association Study; MeDALL: Mechanisms of the Development of Allergy; SNP: single nucleotide polymorphism; LD: linkage disequilibrium. Results in bold are significant associations. [#]: A2 was used as the reference allele; [¶]: LD block annotation is described in the supplementary material; [+]: results from MeDALL meta-analysis; [§]: results from meta-analysis in DAG and MeDALL cohorts; [f]: SNPs previously found to be associated with asthma [2, 9, 10].

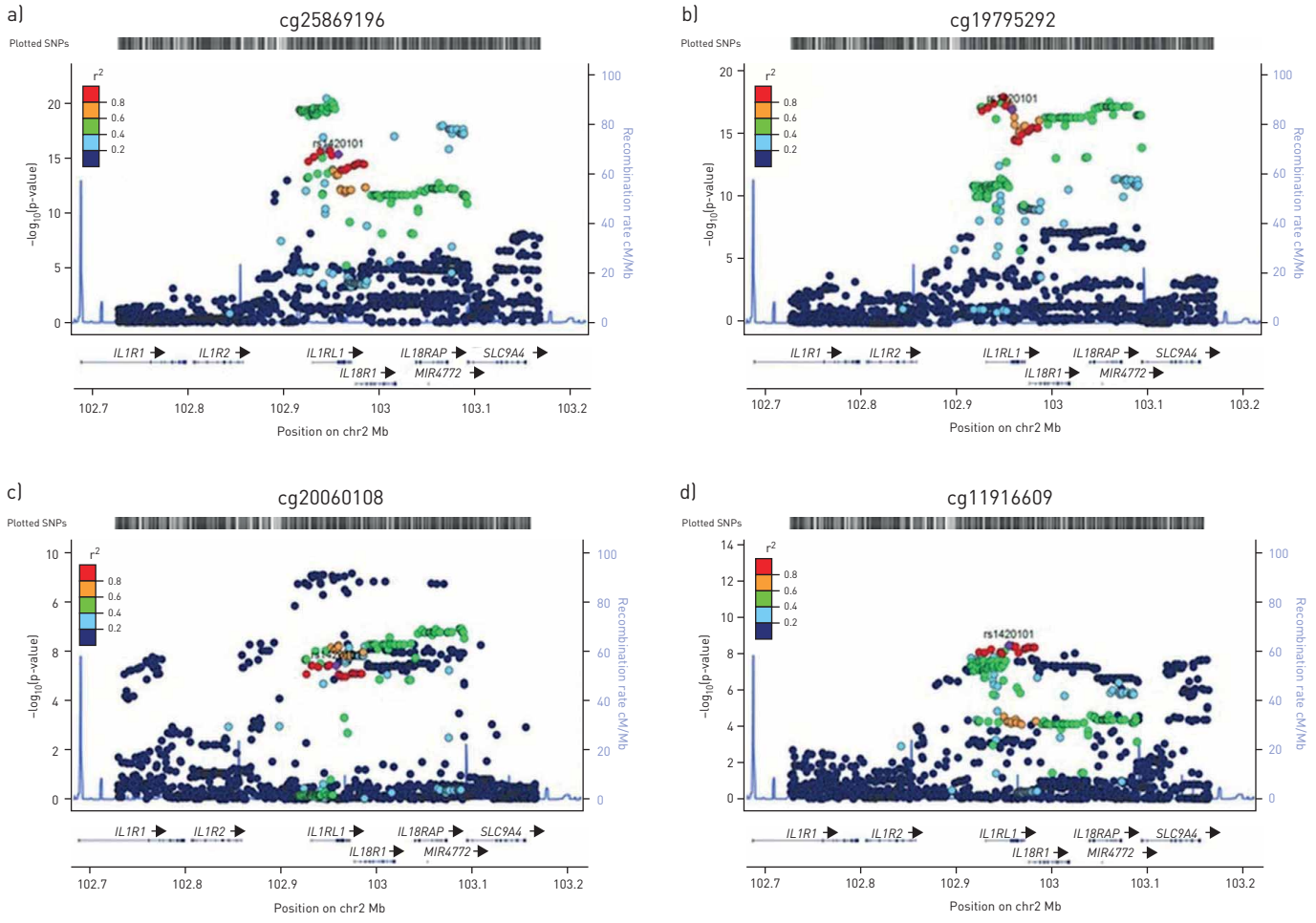

FIGURE 2 Regional association plots showing −log10 p-values for the cell-type-corrected association between single nucleotide polymorphisms (SNPs) in the *interleukin-1 receptor-like 1* (*IL1RL1*) genomic region and *IL1RL1* 5′-C-phosphate-G-3′ (CpG) sites a) cg25869196, b) cg19795292, c) cg20060108 and d) cg11916609 in the MeDALL meta-analysis. The colour of the SNPs is representative of the linkage disequilibrium with rs1420101 (purple circle) with the r^2 scale ranging from 0 to 1.

Methylation in the IL1RL1 *region is associated with cis-meQTLs*

The selected *IL1RL1* region included 47 CpG sites with nine CpG sites in the gene body of *IL1RL1*. SNPs in five different LD blocks were significantly associated with methylation in four CpG sites in the *IL1RL1* gene body at age 4 years (top hits: rs76886731/cg25869196, $p=2.91\times10^{-21}$; rs1420104/cg19795292, $p=1.20\times10^{-18}$; rs56179005/cg20060108, $p=5.08\times10^{-13}$; rs1420101/cg11916609, $p=6.88\times10^{-7}$) (figure 2a–d). These four CpG sites are located in the distal promoter (cg11916609), intron 1A (cg19795292 and cg25869196) and intron 1B (cg20060108). The T allele of rs1420101 was significantly associated with lower methylation levels at all four *IL1RL1* CpG sites (cg25869196, $p=3.73\times10^{-16}$; cg20060108, $p=5.18\times10^{-8}$; cg11916609, $p=6.88\times10^{-7}$; cg19795292, $p=1.20\times10^{-17}$), and was also associated with CpG methylation in *IL1RL2*, *IL18RAP* and *SLC9A4* (supplementary table S3). We also identified strong *IL1RL1* meQTLs in *IL1R1*, *IL1RL2*, *IL18R1*, *IL18RAP* and *SLC9A4* (supplementary table S4), but found no trans-meQTLs.

IL1RL1 *SNPs strongly regulate serum IL1RL1-a levels*

GWAS on IL1RL1-a serum levels in the DAG and PIAMA cohorts showed that *IL1RL1* SNPs are strong cis-pQTLs (top associated SNP rs13020553: DAG beta= −0.33, $p=5.2\times10^{-36}$; PIAMA beta= −0.12, $p=1.45\times10^{-15}$) (figure 3a–c). Eight significant trans-pQTLs were identified in PIAMA, but were not replicated in DAG (supplementary table S5) and the meta-analysis yielded no significant trans-pQTLs. Meta-analysis of *IL1RL1* SNPs in the DAG and MeDALL cohorts provided even stronger evidence for highly significant cis-pQTLs. In all combined cohorts, the T allele of rs142010 was associated with lower IL1RL1-a serum levels ($p=2.83\times10^{-56}$).

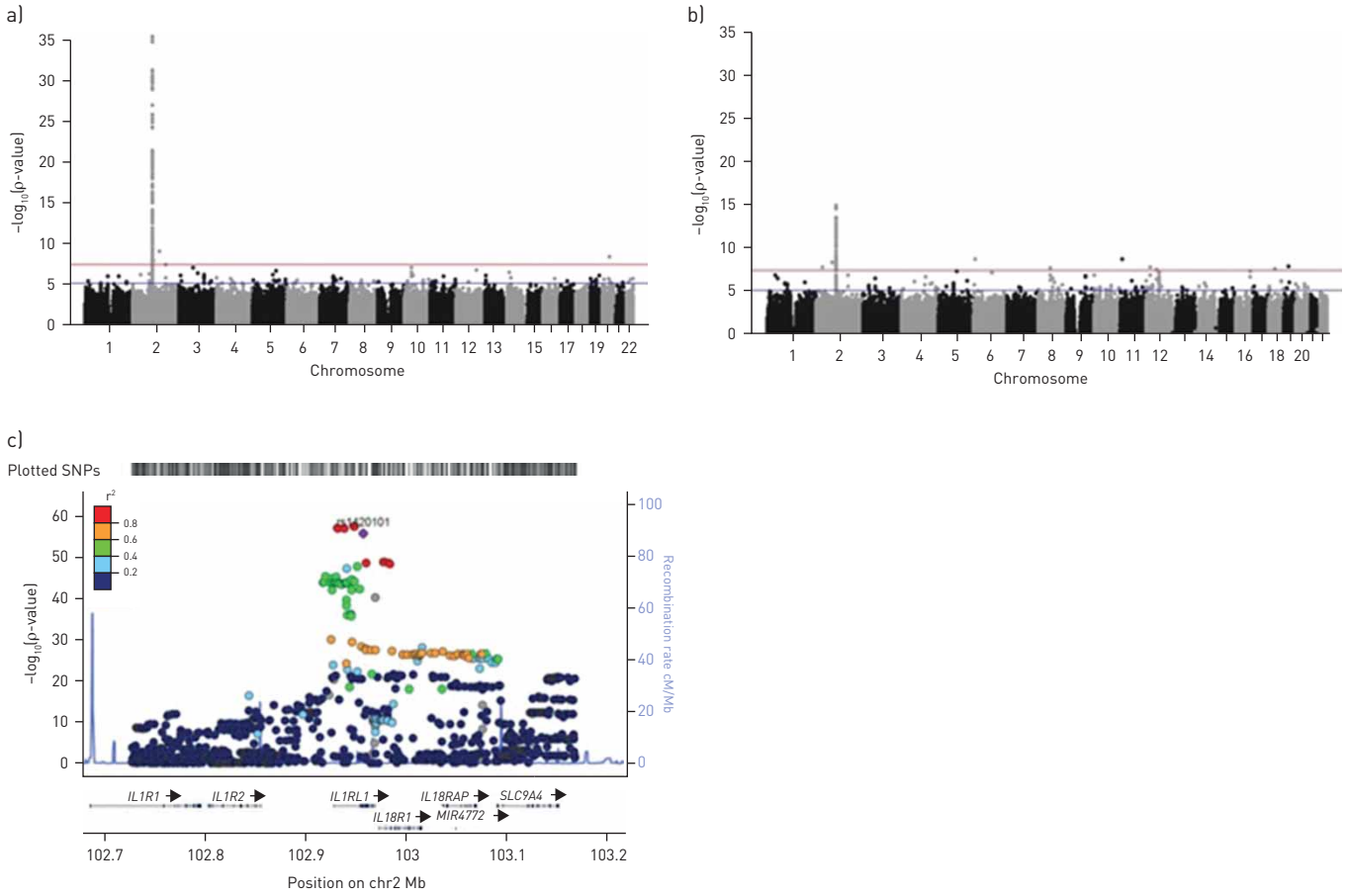

FIGURE 3 Association between single nucleotide polymorphisms (SNPs) and serum interleukin-1 receptor-like 1 (IL1RL1)-a levels. Manhattan plots show results of genome-wide association studies in a) the DAG cohort and b) the PIAMA cohort. The red line indicates the genome-wide significance threshold of a p-value of 5×10^{-8}; the blue line indicates a less stringent p-value of 1×10^{-5}. c) A regional association plot shows results of the *IL1RL1* genomic region meta-analysis in the DAG and MeDALL cohorts. The colour of the SNPs is representative of the linkage disequilibrium with rs1420101 (purple circle) with the r^2 scale ranging from 0 to 1.

Conditional analysis in DAG showed that three independently associated SNPs (rs1420101, rs11685424 and rs13015714), together with age and sex, explained 42% of the variation in IL1RL1-a serum levels (table 3). LD values between the SNPs tagging the different LD blocks are presented in supplementary table S6.

TABLE 3 Multivariate model explaining 42% of the variation in interleukin-1 receptor-like 1 (IL1RL1)-a levels in the DAG cohort

Variable	Position	Gene	Location	A1	A2#	MAF	Beta	SE	p-value
rs1420101	102957716	*IL1RL1*	Exon 5E	C	T	0.39	−0.23	0.03	4.1×10^{-9}
rs11685424	102926981	*IL1RL1*	Distal promoter	G	A	0.49	−0.08	0.04	0.03
rs13015714	102971865	*IL18R1*	5′ flanking	T	G	0.22	0.06	0.03	0.04
Sex							−0.39	0.3	2.8×10^{-32}
Age							0.006	0.001	3.64×10^{-8}

DAG: Dutch Asthma Genome-wide Association Study; MAF: minor allele frequency. #: A2 is the minor allele and was used as the reference allele. Results are from conditional analyses on single nucleotide polymorphisms (SNPs) selected from the four linkage disequilibrium (LD) blocks most strongly associated with IL1RL1-a levels (rs1420101, rs11685424, rs13015714 and rs1035130). A multivariate model with a backward stepwise regression analysis was used, which showed that an independent effect remained with SNPs from three LD blocks. This model, with adjustment for age and sex, explained (predicted) 42% of serum IL1RL1-a levels in the DAG cohort.

TABLE 4 Associations of serum interleukin-1 receptor-like 1 (IL1RL1)-a levels with asthma and asthma-related phenotypes in the DAG cohort

Variable	n	Beta (95% CI)	p-value
Asthma	573 (469 cases)	−0.06 (−0.15–0.039)	0.24
Age years	573	**0.007 (0.00–0.05)**	**2.25×10^{-7}**
Sex	573 (249 males)	**−0.36 (−0.43–−0.29)**	**5.36×10^{-22}**
FEV$_1$ (%pred)[#]	441	0.001 (−0.00–0.00)	0.32
Total IgE kU·L^{-1}[#]	468	0.00 (0.00–0.01)	0.5
Atopy[#¶]	457 (390 cases)	0.08 (−0.03–0.19)	0.16
Eosinophils 10E7·L^{-1}[#]	456	**−0.06 (−0.10–−0.01)**	**0.02**

Linear regression analysis was used to test for association between serum IL1RL1-a levels (ng·mL^{-1}) and asthma or asthma-related phenotypes in DAG. Beta and 95% CI are calculated on log transformed IL1RL1-a levels. Text in bold indicates p<0.05. DAG: Dutch Asthma Genome-wide Association Study; FEV$_1$: forced expiratory volume in 1 s. [#]: asthma-related phenotypes in the DAG cohort only investigated in asthma patients, corrected for age and sex; [¶]: atopy is defined as a positive response to one or more intracutaneous or skin prick tests.

Association of IL1RL1 *methylation with asthma or IL1RL1-a levels*

A candidate CpG meta-analysis of the association between nine *IL1RL1* CpG sites and asthma revealed one nominally significant CpG site located in the distal promoter, cg17738684 (beta= −0.006, p=0.02), but this finding lost significance when correcting for blood cell composition and multiple testing (supplementary table S7). An epigenome-wide association study on IL1RL1-a levels revealed two trans-CpG sites, cg26748568 (chr 16, intergenic region, $p=2.70\times10^{-08}$) and cg08889789 (chr 4, exon 2 *Macrophage Erythroblast Attacher* (*MAEA*), $p=8.93\times10^{-08}$), to be significantly associated with IL1RL1-a levels (supplementary figure S2).

IL1RL1 *SNPs do not regulate IL1RL1-a levels* via *methylation*

We next performed causal inference testing [34] on *IL1RL1* methylation and the protein-associated SNP rs1420101 with cg11916609, cg19795292, cg25869196, cg20060108 and serum IL1RL1-a levels in PIAMA at 4 years of age. There were independent relationships between the SNP and methylation, and between the SNP and IL1RL1-a levels, indicating that our strongest pQTL *IL1RL1* SNP, rs1420101, is not regulating protein levels through methylation of these selected CpG sites (supplementary table S8).

IL1RL1-a levels are associated with eosinophils and allergic sensitisation, but not asthma

No significant differences in IL1RL1-a protein levels in serum were found between asthma cases and controls in the DAG and MeDALL cohorts, but IL1RL1-a levels were associated with sex and age (table 4).

TABLE 5 Associations of serum interleukin-1 receptor-like 1 (IL1RL1)-a levels with asthma and asthma-related phenotypes in the MeDALL cohorts

Variable	PIAMA			BAMSE			INMA		
	n	Beta (95% CI)	p-value	n	Beta (95% CI)	p-value	n	Beta (95% CI)	p-value
Asthma	632 (70 cases)	0.001 (−0.06–0.06)	0.97	184 (42 cases)	0.09 (−0.11–0.3)	0.36	285 (49 cases)	0.03 (−0.08–0.14)	0.55
Age years	666	**0.01 (0.00–0.02))**	**0.007**						
Sex	666 (373 males)	0.02 (−0.17–0.06)	0.26	248 (134 males)	−0.03 (−0.18–0.12)	0.68	248 (134 males)	−0.04 (−0.12–0.04)	0.33
Total IgE kU·L^{-1}	664	0.00 (−0.01–0.01)	0.93						
Sensitisation[#]	663 (264 cases)	0.01 (−0.02–0.05)	0.56	248 (59 cases)	**0.21 (0.03–0.38)**	**0.02**			
Eosinophils 10E7·L^{-1}	274	0.001 (−0.03–0.03)	0.94						

Linear regression analysis was used to test for association between serum IL1RL1-a levels (ng·mL^{-1}) and asthma or asthma-related phenotypes in MeDALL cohorts. Beta and 95% CI are calculated on log transformed IL1RL1-a levels. Text in bold indicates p<0.05. MeDALL: Mechanisms of the Development of Allergy; PIAMA: Prevention and Incidence of Asthma and Mite Allergy; BAMSE: Children/Barn, Allergy, Milieu, Stockholm, an Epidemiological survey; INMA: Infancia y Medio Ambiente. [#]: a child was considered to be sensitised if at least one of the available specific IgE to aero or food allergens had a value ⩾0.35 kU·L^{-1}.

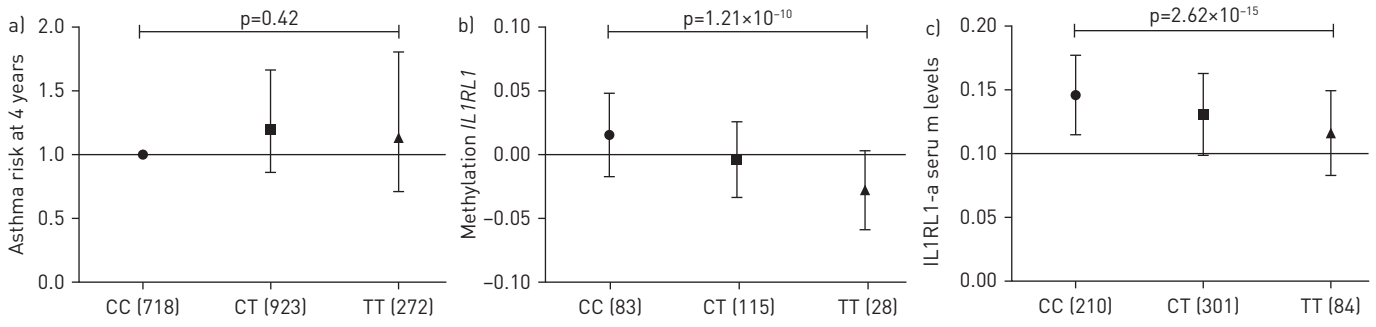

FIGURE 4 Results of association analyses of rs1420101 with a) risk of asthma at age 4 years, b) cell-corrected *interleukin-1 receptor-like 1* (*IL1RL1*) methylation of 5′-C-phosphate-G-3′ site cg19795292 and c) serum IL1RL1-a levels in the PIAMA cohort. Results are displayed per genotype of rs1420101. Data in a are presented as OR (95% CI). Data in b and c are presented as mean±SD. The p-value in each association analysis was calculated using an additive model.

In DAG, higher levels of IL1RL1-a correlated with lower blood eosinophil counts (p=0.02) in asthma patients. IL1RL1-a levels were significantly higher in sensitised children when compared to non-sensitised children in BAMSE (p=0.02) (table 5).

Discussion

A comprehensive analysis of the complex relationship between SNPs, methylation sites and protein levels of *IL1RL1* showed that asthma-associated *IL1RL1* SNPs strongly affected gene methylation and serum IL1RL1-a protein levels. *IL1RL1* methylation was not associated with IL1RL1-a levels. Furthermore, we did not observe a strong association between *IL1RL1* methylation and *IL1RL1* protein expression with asthma.

Our study is the first to interrogate the role of *IL1RL1* gene methylation in asthma. We found that methylation levels at four *IL1RL1* CpG sites in whole blood DNA were associated with *IL1RL1* SNPs in five different LD blocks, irrespective of cell type composition. This is relevant, because *IL1RL1* SNPs were previously reported to be associated with peripheral blood eosinophil counts [10]. We hypothesised that *IL1RL1* methylation or genome-wide CpG methylation may be associated with asthma and/or serum IL1RL1-a levels. We found no evidence of an association with asthma but we did identify two trans-CpG sites, cg26748568 and cg08889789, that were significantly associated with IL1RL1-a levels. The latter CpG site is located in *MAEA*, a gene encoding a protein that mediates the attachment of erythroblasts to macrophages. Its role in regulating IL1RL1-a is not known and should be further studied.

SNPs in four LD blocks in *IL1RL1* had highly significant strong effects on serum IL1RL1-a protein levels. In the PIAMA cohort we also observed trans-pQTLs, but given that the associated SNPs had a low MAF (0.01) and were not replicated in DAG, we suspect these to be false positive results. In our adult cohort we identified multiple genetic signals that independently regulate protein expression, with three SNPs (rs1420101, rs11685424 and rs13015714) from different LD blocks explaining more than 40% of the IL1RL1-a variation in serum. This adds to the growing body of evidence that the *IL1RL1* locus harbours different, independent genetic signals [15].

For greater insight into the complex extended *IL1RL1* region, we will discuss the LD blocks most strongly associated with gene methylation and IL1RL1-a levels.

The first LD block, centred around rs1420101, was strongly associated with *IL1RL1* CpG methylation at age 4 years and with serum IL1RL1-a levels (figure 4a–c). Rs1420101 was first reported to regulate blood eosinophil numbers and serum IgE, with the T allele leading to a higher eosinophil count and higher IgE levels. The T allele has also been found to be a risk variant for asthma in candidate gene studies [10, 15]. rs1420101(T) is also associated with lower *IL1RL1* mRNA expression levels in airway epithelial cells [35] and lung tissue [35, 36], and lower IL1RL1-a protein levels in BAL fluid. The SNP is in complete LD with rs950880, the most significant SNP found in a previous large GWAS on IL1RL1-a levels [37]. We found that the T allele of rs1420101 was associated with less *IL1RL1* methylation and lower serum IL1RL1-a levels (table 2 and figure 4a–c), with rs1420101 solely explaining 18% of the variation in the protein levels. Given that IL1RL1-a serves as an IL-33 antagonist, lower IL1RL1-a levels might lead to more pronounced allergic inflammation, which is in agreement with published findings that the T allele is associated with a higher asthma risk [10]. In addition, an association has also been found between the T allele and a type-2 high phenotype in asthmatic patients [35]. Rs1420101 is located in intron 5 of IL1RL1-a and -b, and in exon 5E of the IL1RL1-c transcript variant. This *IL1RL1* variant is expressed in human T-helper cell

clones and in a human leukemic cell line, UT-7 [17]. IL1RL1-c localised to the plasma membrane in overexpression studies in COS7 cells, indicating a possible function as a transmembrane receptor [18]. However, IL1RL1-c lacks the intracellular signalling domains present in IL1RL1-b owing to a premature stop codon; therefore, it might not act as a functional IL-33 receptor. We suggest that more research should be focused on this *IL1RL1* isoform.

The second LD block was tagged by SNPs in the distal (rs11685424) and proximal promoter (rs12712141), the 5′ region, and intron 1A of *IL1RL1*. This block was associated with methylation of the distal promoter (cg11916609) and intron 1A (cg19795292 and cg25869196) and with protein *IL1RL1* levels (table 2). Associations of this LD block with blood eosinophils [25], IL1RL1-a BAL levels [26] and serum levels [25, 37], but not with asthma, have previously been reported.

A third LD block contained two distal promoter SNPs, rs6543115 and rs6543116. SNPs in this block were strongly associated with methylation of the distal promoter (table 2). Remarkably, these SNPs were less strongly associated with IL1RL1-a levels. It would therefore be interesting to investigate the effect of these SNPs, compared to that of the aforementioned rs1420101, on the IL1RL1-b transmembrane receptor isoform.

SNPs located in the genes downstream of *IL1RL1* were overrepresented in the fourth LD block, which did not contain the most significantly associated SNPs in the meQTL and pQTL analyses.

Most previously reported asthma-associated SNPs were located in LD block 5, including three IL1RL1-b non-synonymous coding SNPs in exon 11 [2, 9, 10]. The asthma-protective alleles from SNPs in this LD block showed a relatively modest association with *IL1RL1* methylation and IL1RL1-a serum levels (table 2). These data suggest that the association of this LD block with asthma could stem from altered protein function [26, 37] rather than from regulation of (epi)genetic signals, although the latter cannot be excluded given the observed associations. In supplementary table S9 we report the results of other previously reported *IL1RL1* asthma-associated SNPs. Interestingly, some of the asthma-associated signals are not located in the LD blocks most strongly associated with methylation or protein levels. This suggests the presence of alternative mechanisms not mediated by regulation of the studied CpG sites or *IL1RL1* protein isoform.

Using data from the GTEx consortium [38], we found that *IL1RL1* SNPs located in the five LD blocks accounting for the most independent association signals with gene methylation and IL1RL1-a levels in the region considered were also strong expression quantitative trait loci (eQTLs) in whole blood and lung tissue for genes located in the *IL1RL1* region (supplementary table S10). SNP alleles that were associated in our own cohorts with lower levels of IL1RL1-a protein, *e.g.* rs1420101, were also associated with lower *IL1RL1* expression in lung tissue. Moreover, using GTEx data, we found no trans-eQTLs for *IL1RL1* in the lungs, highlighting the importance of polymorphisms at the *IL1RL1* locus on chromosome 2q12 in regulating *IL1RL1* gene expression.

We did not find an association between serum IL1RL1-a levels and asthma in children or adults in our well-powered analyses. This is in contrast to earlier, smaller studies reporting higher serum IL1RL1-a levels in adult atopic asthma patients than in healthy controls [39] or during an acute asthma attack in children [14]. Our findings are, however, in agreement with previous reports on the PIAMA cohort [25] and on patients with severe asthma [26]. Because our analysis was carried out using data from patients with stable asthma not experiencing an exacerbation or recent exacerbation, we speculate that asthma is not associated with serum IL1RL1-a levels but that this may change during exacerbations. Our data from asthma patients confirms that increased serum IL1RL1-a levels are associated with reduced peripheral blood eosinophil numbers [14, 25], consistent with a protective effect of IL1RL1-a on eosinophilic inflammation. Blockade of the *IL-33–IL1RL1* pathway may therefore be considered a possible future therapeutic option for asthmatic patients with eosinophilic Th2-associated inflammation.

We identified nominal significant genetic associations of IL1RL1 region SNPs with asthma in our adult and child cohorts, with an effect size and direction that was in line with previously reported findings in large GWAS [2, 3, 8–16]. This modest association in our cohort might contribute to a limited power for detecting an association between methylation at the IL1RL1 locus or IL1RL1 protein levels and asthma. The fact that the association between IL1RL1 SNPs and asthma lost significance after correction for multiple testing may be due to the relatively low sample size of our study when compared to large-scale GWAS [15]. However, another important factor that could play a role is the large heterogeneity of asthma. There are multiple sub-phenotypes of asthma which we could not properly distinguish in our cohorts. As mentioned before, multiple studies have shown that the IL-33–IL1RL1 pathway is important in type 2 inflammation [4, 5, 26, 35]. The fact that we investigated a general asthma phenotype, as frequently used in population-based epidemiological studies, could explain why we did not find a strong association between IL1RL1 methylation and asthma and between IL1RL1 protein levels and asthma. This supposition

is supported by a recent study in wheezing children aged 2–3 years, which showed that serum IL1RL1-a levels were not associated with doctor-diagnosed asthma at age 6 years, but nevertheless predicted asthma with an increased level of FeNO, a marker for eosinophilic airway inflammation [40]. Finally, SNPs may explain a large proportion of methylation and gene expression, but only very little variation in the ultimate disease phenotype. Based on recent estimations of the genetic heterogeneity of asthma, hundreds of genes may be important in asthma. It is therefore difficult to make causal inferences on functional SNPs in asthma using this approach.

Some limitations of our study need to be addressed. First, we identified separate blocks of SNPs by inspecting the LD in the region, but realise that some correlation between SNPs in different LD blocks is still present (supplementary table S7). Conditional analysis on IL1RL1-a levels, however, confirmed the independence of effects of SNPs in three LD blocks. Second, in our mediation analysis we investigated four *IL1RL1* CpG sites, but other CpG sites that were not quantified or analysed could also be important. Third, our study mainly focused on IL1RL1-a protein, but to fully understand the role of the *IL1RL1* gene in asthma, other receptor variants, *e.g.* IL1RL1-b and -c, should also be investigated. Because we did not find a strong association between SNPs/IL1RL1-a and asthma, causal pathway analyses could not be performed.

In summary, our study identified highly significant associations of *IL1RL1* SNPs with gene methylation and protein expression, as well as identifying multiple independent, functional genetic signals in this gene and gene region. Our analyses suggest, however, that *IL1RL1* methylation is not important for protein expression and that the identified effects of asthma-associated SNPs on methylation and IL1RL1-a levels are not related to asthma (supplementary figure S3). Future research should also focus on the other *IL1RL1* isoforms, on other functional effects of protein-coding variants in the *IL1RL1* gene (region), and on identifying the specific asthma phenotypes for which *IL1RL1* is important, which will lead to diagnostic and personal therapeutic interventions in asthma.

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