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Innate IL-23/Type 17 immune responses mediate the effect of the 17q21 locus on childhood asthma

Running title: Innate immunity, 17q21 loci and childhood asthma

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Key Messages :

- We examined whether innate immune responses explain the genetically inferred increased risk of childhood asthma and exacerbations.

- The effect of variants in the 17q21 locus was mediated by immune mediators induced by ligands mimicking intracellular bacterial and viral colonization.

- The Th17 and innate lymphoid cell type 3-amplifying cytokine IL-23 was the most prominent cytokine involved.

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Abstract:

Background: Several childhood asthma risk loci that relate to immune function have been identified by genome-wide association studies (GWAS), but the underlying mechanisms remain unknown. **Objective:** Here, we examined whether perturbed innate immune responses mediate the association between known genetic risk variants and development of childhood asthma.

Methods: Peripheral blood mononuclear cells from 336 six-month-old infants from the Copenhagen Prospective Studies on Asthma in Childhood ($COPSAC_{2000}$) cohort were stimulated in vitro with six different innate ligands (LPS, CpG, poly(I:C), R848, HDMAPP, and aluminum hydroxide together with low levels of LPS) followed by quantification of 18 released cytokines and chemokines 40h after the stimulations. The innate immune response profiles were decomposed by principal component (PC) analysis and PC1-5 were used in mediation analyses of the effect of 25 known genetic risk variants on childhood asthma until age 7.

Results: The effects of two variants from the 17q21 locus (rs7216389, rs2305480) on asthma and exacerbation risk were significantly mediated by immune parameters induced in response to ligands mimicking intracellular colonization; bacterial DNA (CpG) and double-stranded viral RNA (poly(I:C)). The Th17 and innate lymphoid cell type 3-amplifying cytokine IL-23 was the most prominent cytokine involved.

Conclusion: The 17q21 effect on childhood asthma and exacerbations was partly mediated by deregulation of IL-23 in response to intracellular microbial ligands, which may suggest ineffective clearance of intracellular pathogens in the lungs.

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Manuscript text:

Introduction

Heritability explains 50-80% of asthma risk and is particularly high for childhood onset asthma.[1,2] Several genetic risk loci and their lead single-nucleotide polymorphisms (SNPs) have been identified by independent genome-wide association studies (GWAS).[3–6] A recent consortium-based meta-GWAS study confirmed multiple risk loci for asthma and suggested their involvement in immune regulation by demonstrating enrichment in the enhancer regions of immune cells.[7] Possible biological mechanisms underlying some of these associations have been suggested by functional studies, including altered metabolic pathways and aberrant expression of immune mediators.[8] However, it remains unknown how the most replicated genetic variants in childhood asthma lead to an increased risk of asthma in childhood.

In the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) birth cohort, we collected peripheral blood mononuclear cells (PBMCs) at age 6 months and subsequently quantified the innate immune response by measuring supernatant levels of cytokines and chemokines secreted in response to in vitro stimulations with five different bacterial and viral ligands, as well as the vaccine adjuvant aluminum hydroxide. In this study, we aimed to investigate whether the early life innate immune response represented by these six different signaling pathways mediates the relationship between 25 of the strongest childhood asthma susceptibility variants[6] and development of childhood asthma and lung function traits in the first 7 years of life.

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Methods and Materials

Study cohort

This study is part of the Danish $COPSAC_{2000}$ prospective clinical mother-child cohort study of 411 children born to asthmatic mothers during 1998-2001.[9,10] The children were enrolled at age 1 month and were followed at the COPSAC clinical research unit with scheduled 6-monthly visits until age 7 years as well as at incidences of acute respiratory symptoms.[11,12] The diagnosis and treatment of any airway, allergic or skin symptoms were strictly adhering to predefined, validated algorithms.[11,12] The study was approved by the Copenhagen Ethics Committee (KF 01-289/96) and the Danish Data Protection Agency (2002-41-2434) and followed the principles of the Declaration of Helsinki. Written and oral informed consent was obtained from the parents at enrolment.

Stimulation of PBMCs

To fully categorize immune responses from different innate immune pathways, peripheral blood mononuclear cells from 336 six-month-old infants from the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC2000) cohort were stimulated in vitro with six different innate ligands (LPS, CpG, poly(I:C), R848, HDMAPP, and aluminum hydroxide together with low levels of LPS) followed by quantification of 18 released cytokines and chemokines 40h after the stimulations (see **online methods** for detail).[13,14] The selected six different ligands represent a wide variety of pathogen associated molecular patterns (PAMPs), from bacterial extracellular component LPS to intracellular DNA (CpG) and RNA component specific for bacterial and viruses (poly(I:C), R848), aiming to turn on different innate immune signaling pathways via membrane bound pathogen recognition receptors (PRRs). In addition to the membrane bound PRRs, the activation of cytosolic PRRs was enabled by including aluminum hydroxide and low levels of LPS. Besides the PAMPs, HDMAPP, a ligand known to activate γδT cells, was also included to get an idea of the immune response from γδT cells. After 40h of the stimulations, the level of IFN-γ, IL-12p70, CXCL10, IL-18, IL-4, IL-5, IL-13, IL-31, CCL17, CCL24, IL-1β, IL-6, IL-17A, IL-23, CCL20, CXCL8, IL-22, CCL27 and IL-10 were quantified.

Asthma risk loci

Based on a recent meta-GWAS and other GWAS studies[3,4,6,7], we selected 25 top risk SNPs for childhood asthma (see selected SNPs in **online methods**). Allelic discrimination of those SNPs in

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$COPSAC_{2000}$ was investigated by genome-wide genotyping using the Infinium HumanOmniExpressExome BeadChip Kit (Illumina, San Diego, Calif). Sample and marker quality controls were performed as previously reported[7] (see **online methods**).

Clinical endpoints

The clinical endpoints included asthma, transient and persistent asthma, asthma exacerbations, lower respiratory tract infections (LRTIs) and lung function measured by spirometry (FEV1, MMEF), plethysmography (sRaw) and bronchial responsiveness to methacholine (PD20) (see **online methods**).

Covariates

Covariates included a composite score for social circumstances (first principle component (PC1) of a PC analysis (PCA) of maternal educational level, annual family income, and maternal age); sex; older siblings; delivery mode; cat exposure at birth [15] ; parental smoking and passive smoking [16]; antibiotic use during pregnancy; and parental history of eczema and allergy.

Statistical analysis

Data transformation

The raw concentrations of immune mediators were adjusted for background levels and transformed before decomposed by PCA. The first five PCs were then used in the following analysis by considering the synergistic effects among the cytokines (see **online methods**).

Models for SNPs

Associations between genotypes and the clinical phenotypes were examined using linear, logistic, quasi-Poisson, and cox proportional hazard regression models based on the type of clinical endpoint. Genotypes with significant effect on at least one clinical endpoint ($p<0.05$) were selected for subsequent mediation analysis. All SNPs were analyzed by an additive model (0, 1 or 2 risk alleles), except for the two lead SNPs from the 17q21 locus, which were examined by a recessive model according to findings in the $COPSAC_{2000}$ cohort.[17]

Mediation analysis

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Our current work was mainly built upon causal mediation analysis generated by a mediation package for R.[18,19] This package was written according to the potential outcome framework using a simulation-based method consisting of two regression model-based equations for estimating the causal mediation effect.[20] It uses accelerated failure time (AFT) models for age at onset data with negative estimate suggests a higher risk of disease, i.e. less time to disease onset. The total effect of the selected SNP on the specific clinical outcome was decomposed into a direct effect (Average Direct Effects - ADE), which is independent of the immune responses, and an indirect effect (Average Causal Mediated Effects - ACME), which is mediated by the immune responses. Hence, ACME was the term we were interested in. The percentage explained by the immune response was calculated as ACME/total effect.

Bias from confounders were adjusted for in the corresponding regression-based models in the mediation analysis. Potential confounders were selected from covariates based on their associations to immune profiles and clinical endpoints. Those confounders were subsequently included in the mediation analysis to obtain adjusted mediation effects.

The mediation analyses were based on regression models with 5000 nonparametric bootstrap simulations to assure robustness of the results. Confidence intervals of mediation estimates were bias corrected and reported as the measure of significance, instead of p-value. Due to the missingness in relevant covariates, multiple imputation was employed when performing adjusted mediation analysis. The confidence interval of adjusted mediation estimates was obtained by taking the 95-percentile of the bootstrap results from the multiple imputed datasets.
All analyses were performed in R 3.6.

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Results

Baseline characteristics

A total of 336 (82%) of the 411 infants in the $COPSAC_{2000}$ cohort had both genotyping data and PBMCs collected at age 6 months, the latter being successfully stimulated with the five different viral and bacterial ligands or aluminum hydroxide (**Online Table E1).**

Among the 336 infants, 62 (18.5%) developed asthma at some point in their first 7 years of life: 25 (7%) had transient asthma and 37 (11%) had persistent asthma. At age 6 years, 225 (67%) of the 336 children had available PD20 data, 257 (76%) had spirometry assessments of FEV1 and 254 (76%) had MMEF data, and 255 (76%) had measurements of airway obstruction determined by sRaw. Acute asthma exacerbations occurred in 53 (16%) of the children before age 7 years. LRTI before age 3 was recorded in 165 (56%) of children.

Baseline characteristics are given in **Online Table E2**, showing no significant differences between the 336 children included in the study and the 75 excluded children.

SNPs, innate immune profiles and clinical phenotypes

First, we examined the association between the selected asthma risk SNPs and the clinical phenotypes (SNP allele frequencies are shown in **Online Table E3)**. Several of the selected SNPs were associated with the clinical phenotypes (**Figure 1**). Particularly, SNPs in the 17q21 locus, rs7216389 and rs2305480, were associated with an increased risk of asthma in the first 7 years of life: rs7216389, HR=1.80 [1.12, 2.90], p=0.01 and rs2305480, HR=1.73 [1.04, 2.86], p=0.03. In addition, these SNPs were associated with transient asthma: rs7216389, OR=2.60 [1.16, 5.82], p=0.02 and rs2305480, OR=2.78 [1.15, 6.72], p=0.02, increased number of acute asthma exacerbations: rs7216389, IRR=2.23 [1.17, 4.27], p=0.02 and rs2305480, IRR=2.15 [1.03, 4.47], p=0.04, and increased number of LRTIs: rs7216389, IRR=1.50 [1.13, 1.99], p=0.01.

Second, we analyzed the associations between asthma risk SNPs and the ligand-stimulated immune profiles captured in PC1-5, restricting the analyses to SNPs showing significant effects on at least one of the clinical phenotypes from the association analysis described above. Several statistically significant relationships were observed as shown in **Figure 2A**. Of note, both 17q21

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variants were significantly associated with the poly(I:C)-stimulated immune profile captured in PC3, and rs7216389 was further associated with the CpG-stimulated immune profile in PC3.

Third, we investigated associations between the ligand-stimulated innate immune profiles and the clinical phenotypes (**Figure 2B**), with a particular interest in immune profiles associated with variants in the 17q21 locus, such as poly(I:C)-PC3 and CpG-PC3. These two immune profiles were strongly associated with development of asthma in the first 7 years of life: HR_{CpG}=1.50 [1.21, 1.86], p_{CpG}<0.01; $HR_{poly(I:C)}$=0.76 [0.61, 0.94], $p_{poly(I:C)}$=0.01. In addition, CpG-PC3 was also associated with other asthma-related phenotypes (**Figure 2B**). Further, PC2 from R848 related to rs2305480 was also associated with asthma development: HR_{R848}=0.84 [0.71, 0.98], p_{R848}=0.03 (**Figure 2B**). In the following mediation analysis, we investigated the mediation effect of each immune profile for significant SNPs on clinical phenotypes. For the two SNPs from 17q21 locus, a combined risk score based on the number of risk alleles was calculated [21]and followed with a mediation analysis, which showed a similar but less significant result comparing to that of the individual SNPs (**Table E4**).

Mediation analysis

Effect of genotype on asthma development mediated via the innate immune responses

The mediation analysis for asthma was based on an AFT model where a negative mediation effect indicated a higher risk. This analysis suggested that enhanced risks of both 17q21 variants on asthma were mediated by immune profiles captured in both CpG and poly(I:C) for PC3 (**Figure 3** and **Table 1**). The effect of rs7216389 on asthma was mediated by CpG-PC3 ($ACME_{CpG}$= -5.39 [-25.02, -1.30]) and poly(I:C)-PC3 ($ACME_{poly(I:C)}$= -2.94 [-13.58, -0.63]), where each explained 18.4% and 12.1% of the increased risk. The effect of rs2305480 on asthma were mediated by the same immune profiles as above: CpG-PC3 ($ACME_{CpG}$= -4.68 [-25.15, -0.77]) and poly(I:C)-PC3 ($ACME_{poly(I:C)}$= -2.91 [-16.67, -0.43]), corresponding to 15.6% and 11.1% explained variance, respectively. Adjusting the mediation analysis with selected covariates, which were associated with either immune profiles and/or clinical phenotypes (**Online Figure E1**), did not change the outcome of the analysis on rs7216389 (adjusted $ACME_{CpG}$= -5.23 [-18.14, -0.10] and adjusted $ACME_{poly(I:C)}$= -3.90 [-13.83, -0.18]), explaining 17.2% and 13.1% of the increased risk on asthma, respectively (**Figure 3** and **Table 1**). However, for rs2305480, the adjusted mediation effect was

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only captured by poly(I:C)-PC3 (adjusted $ACME_{poly(I:C)}$= -4.52 [-15.96, -0.25]) with 14.3% of the increased risk on asthma explained (**Figure 3** and **Table 1**).

Effect of genotype on exacerbation risk mediated via the innate immune responses

For exacerbations, the mediation analysis was based on a Poisson model, where a positive ACME estimate suggested a higher risk. The PC3 immune profile derived from CpG-stimulated PBMCs had a significant positive mediating effect on risk of exacerbations from both rs7216389 ($ACME_{CpG}$= 0.11 [0.03, 0.30]) and rs2305480 ($ACME_{CpG}$= 0.10 [0.01, 0.30]), and accounted for 18.1% and 15.4% of the risk, respectively (**Figure 3** and **Table 1**). Mediation analysis with confounder adjustments suggested significant mediation effects conveyed by CpG-PC3 for rs7216389 ($ACME_{CpG}$= 0.11 [0.02, 0.27]) and rs2305480 ($ACME_{CpG}$= 0.09 [0.01, 0.24]), each contributed 14.8% and 17.6% of the risk. poly(I:C)-PC3 mediated the effect of rs2305480 on exacerbations ($ACME_{poly(I:C)}$= 0.06 [0.002, 0.21], but became insignificant after confounder adjustment (**Figure 3, Table 1)**.

The drivers of the mediation effects

Focusing on the immune patterns represented by CpG-PC3 and poly(I:C)-PC3, it appeared from the loading plots (**Figure 4**) that the effect of the 17q21 locus risk variants on childhood asthma and exacerbations was mediated by a synergistic interplay between several cytokines. Particularly, enhanced production of IL-23 and IL-31. This led us to further examine the mediation effect of IL-23 and IL-31 individually, by employing the same mediation analysis framework described above. In the meantime, the associations between the pinpointed cytokines (IL-23, IL-31) and clinical outcomes as well as the 2 SNPs at 17q21 locus were also examined. Significant associations with asthma until 7 years and exacerbations were only found for IL-23 (**Table E5**). Consistently, significant mediation effect was also found for IL-23, in line with the result from the immune profiles.

For asthma until 7 years, the effect of rs7216389 and rs2305480 were both mediated by IL-23 in response to CpG ($ACME_{rs7216389}$= -2.28 [-13.10, -0.20], $ACME_{rs2305480}$= -3.07 [-17.59, -0.34]), and explained 9.3% and 11.8% of the increased risk, respectively (**Figure 5** and **Table 2**). The same did not apply for IL-23 in response to poly(I:C) for either variant.

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For asthma exacerbations, IL-23 from CpG stimulation mediated the effect of both rs7216389 (ACME= 0.10 [0.01, 0.32]) and rs2305480 (ACME= 0.08, [0.003,0.34]), each accounting for 15.2% and 14.3% of the increased risk (**Figure 5** and **Table 2**).

Since some children did not produce IL-23 by their PBMCs in response to innate ligands (IL-23 non-responders, number = 72), a sensitivity analysis was done by repeating the mediation analysis after excluding IL-23 non-responders, which showed similar results (**Table E6-9, Figure E2**).

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Discussion

We found that innate immune profiles at age 6 months upon PBMC-stimulation with ligands mimicking bacterial unmethylated DNA (CpG) and viral double-stranded RNA (poly(I:C)) significantly mediated the effect of two lead SNPs in the 17q21 locus on development of childhood asthma and asthma exacerbation until age 7, explaining up to 18% of the increased risk. The cytokine patterns in response to CpG and poly(I:C) stimulations with significant mediation effects were both characterized by immune profiles dominated by elevated IL-23. This deregulated immune response may lead to ineffective clearance of viruses and intracellular bacteria that could explain some of the underlying mechanism related to 17q21 genetic variants.

The CpG ligand which mimics bacterial DNA is a potent stimulus of human Toll like receptor 9 (TLR9).[22,23,24] The poly(I:C) ligand that resembles viral double-stranded RNA is an agonist of TLR3.[23,25,26] Activation of the TLR9 and TLR3 pathways lead to the release of Type 1 interferons and pro-inflammatory cytokines[25], which results in activation of NK cells and cytotoxic T cells and subsequently elimination of intracellular infectious agents by cellular mediated killing of infected host cells. Thus, children with impaired Type 1 immune responses to CpG and poly(I:C) may suffer from bacterial and viral airway colonization or infections that may drive the development of asthma or the symptoms thereof. In line with this we have previously demonstrated association between bacterial colonization and later development of asthma.[11,27] Also, we have found bacteria closely associated with asthma exacerbations.[28]

However, in this study, instead of an increased expression of Type 1 cytokines, we observed immune profiles dominated by enhanced expression of IL-23 in response to CpG and poly(I:C) to mediate the effect of the 17q21 variants on asthma development and exacerbations. For human immune cells, IL-23 is indispensable for promoting expansion of Type 17-associated immune cells including Th17 and type 3 innate lymphoid cells (ILC3) from corresponding precursors as well as for sustaining and expanding the memory Th17 cells.[29] By secreting Type 17 signature cytokines such as IL-17A, IL-22 and GM-CSF, Type 17 immune cells are responsible for clearing off extracellular bacterial and fungal infections, mainly due to stimulation of CXCL8 production by nearby cells, resulting in recruitment of neutrophils and macrophages to the site of infection. Besides the aforementioned Type 17 immune cells, IL-23 in combination with IL-1β can also

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stimulate γδT cells and NKT cells to produce IL-17A.[30] Hence, both innate and adaptive Type 17 immune cells are closely related to enhanced IL-23.

The innate immune response against CpG and poly(I:C) suggests an incorrect immune response represented by a skewed Type 17 immune response mounted by carriers of 17q21 locus risk variants. Based on this we speculate that carriers of 17q21 locus risk variant may hold a predominant Type 17 immune response against viral and intracellular bacterial infections in early life. Such dysfunctioning immune response may lead to recurrent infections and prolonged IL-23 secretion, which further enhance the recruitment of neutrophils and macrophages by supporting Type 17 immune cells differentiation and activation, resulting in development of asthma and asthma exacerbations during childhood due to continued immune activation in the airways.[31,32]

The mediating effect of the type 17 skewed immune profiles suggested by our study is consistent with previous reports, both pointing to a non-atopic pathway of 17q21 locus-associated childhood asthma.[6,17,33] It also helps to shed light on detangling the puzzle of the etiology of severe asthmatics featured by corticosteroid resistance, where type 17 immune responses have been implicated.[34,35,36] The association between a Type 17 based immune profile and 17q21 risk variants suggested by our findings is also in line with a previous study, which showed that children carrying 17q21 risk alleles had elevated IL-17A secretion in their cord blood.[37] We did not identify elevated IL-17A in our study as we focused on innate immune cell activation and harvested the supernatant at 40 hours post-stimulation, which was too early for IL-17A to secrete.[38]

There are several advantages of this study. First, the prospective nature of our study and the storage of PBMCs from age 6 months provide a unique possibility to investigate functional immunological properties in infancy before onset of asthma-related outcomes. As the PBMCs were collected in asymptomatic infants, the innate immune response is unlikely to be affected by ongoing disease-driven inflammation.

Another advantage is that mothers of all infants were asthmatic, Caucasians and lived in the Copenhagen area. This ensures a relatively homogeneous genetic background of the study population, which minimizes the risk of confounding from population stratification. Moreover, the

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children in this study were solely diagnosed and treated by the COPSAC research pediatricians under the same rigid standardized algorithms for any asthmatic symptoms, that ensured high reliability of the clinical outcomes.

Paired with the advantages, the study also has some limitations. A limitation to observational studies and mediation analyses is the risk of residual confounding from lifestyle or host factors. Here, we adjusted for confounding from a range of early-life factors that may impact both the immune response and asthma-endpoints, but we cannot rule out that other environmental or host factors might also contribute to the observed mediation effects. Another limitation is that the levels of cytokines were all measured at 40h after stimulation and all the subsequent analysis were based upon the single time measurement which may not reflect the dynamic nature of the immune response. Moreover, our findings about ineffective clearance of viruses and intracellular bacteria were based on PBMCs and may not be extrapolated to the immune response in airway epithelial cells, which are the typical target of respiratory infections in asthmatics.

Finally, the 17q12-21 region is characterized by extensive linkage disequilibrium with a large number of highly correlated SNPs associated with asthma, and the causal variants in the region remain unelucidated.[8] We chose two different tagging variants in the region. The first variant, rs7216389, was chosen as it is the top SNP in the first large-scale GWAS on childhood asthma [5] and also strongly associated with early asthma and exacerbations in $COPSAC_{2000}$.[17] The other chosen variant, rs2305480, is a non-synonymous variant in GSDMB, which was the strongest associated variant in relation to childhood asthma with early onset in a later GWAS. [6,3] Rs2305480 is closely correlated with a putative causal variant (rs12936231) related to regulation of ORMDL3 expression [39] (R2=0.82) as well as a splice variant (rs11078928, R2=1.0) in GSDMB related to pyroptotic activity in airway epithelium. [40] The two 17q21 tagging variants in our study are also internally correlated (R2=0.80) and as expected all observed associations were quite similar for the two SNPs.

Conclusion

These coupled experimental and clinical data suggest that a deregulated innate immune response, dominated by IL-23, towards intracellular microbes in infants partly explains the increased risk of

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developing asthma and experiencing asthma exacerbations during childhood in carriers of risk variants in the 17q21 locus.

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Author contributions: The guarantor of the study is HB who is responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data and writing of the manuscript. HB had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. NW, SB and BC were responsible for data analysis and wrote the first draft of the manuscript. JML, AHT, MAR, JS and KB contributed to design of the study, interpretation of data and writing of the manuscript. JML, AHT and SB were responsible for generating the immune response data. CTW, SB and MAR supervised the data analytical pipeline developed for transformation of raw cytokine data. All co-authors have contributed substantially to the analyses and/or interpretation of the data and have provided important intellectual input and approval of the final version of the manuscript. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

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Tables:

Table 1. Mediation analysis for asthma.

Asthma				
17q variants	**Innate ligand**	**Estimate(95%CI) unadjusted**	**Estimate(95%CI) adjusted**	**term**
rs2305480	**CpG**	**-4.68(-25.15,-0.77)**	-4.92(17.92,0.71)	Indirect effect(ACME)
		-25.41(-86.67,-1.79)	-27.89(78.82,4.49)	Direct effect
		-30.09(-102.50,-6.89)	-32.82(-89.85,-0.38)	Total Effect
	poly(I:C)	**-2.91(-16.67,-0.43)**	**-4.52(-15.96,-0.25)**	Indirect effect(ACME)
		-23.32(-68.50,-5.29)	-27.62(-69.57,0.44)	Direct effect
		-26.23(-79.46,-7.78)	-32.13(-80.17,-3.62)	Total Effect
rs7216389	**CpG**	**-5.39(-25.02,-1.30)**	**-5.23(-18.14,-0.10)**	Indirect effect(ACME)
		-23.92(-76.38,-4.62)	-25.18(-69.87,6.14)	Direct effect
		-29.31(-92.16,-9.90)	-30.4(-81.66,0.17)	Total Effect
	poly(I:C)	**-2.94(-13.58,-0.63)**	**-3.9(-13.83,-0.18)**	Indirect effect(ACME)
		-21.39(-59.22,-5.21)	-25.87(-65.59,0.50)	Direct effect
		-24.34(-68.29,-7.79)	-29.77(-74.78,-2.70)	Total Effect
Exacerbations				
17q variants	**Innate ligand**	**Estimate(95%CI) unadjusted**	**Estimate(95%CI) adjusted**	**Term**

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rs2305480	**CpG**	**0.10(0.01,0.30)**	**0.09(0.01,0.24)**	Indirect effect(ACME)
		0.55(-0.01,1.27)	0.53(-0.04,1.25)	Direct effect(ADE)
		0.66(0.07,1.42)	0.62(0.04,1.37)	Total Effect
	poly(I:C)	**0.06(0.002,0.21)**	0.06(-0.01,0.17)	Indirect effect(ACME)
		0.50(-0.02,1.25)	0.55(-0.07,1.22)	Direct effect(ADE)
		0.56(0.04,1.36)	0.61(-0.02,1.30)	Total Effect
rs7216389	**CpG**	**0.11(0.03,0.30)**	**0.11(0.02,0.27)**	Indirect effect(ACME)
		0.51(-0.05,1.31)	0.50(-0.07,1.21)	Direct effect(ADE)
		0.62(0.05,1.43)	0.61(0.03,1.36)	Total Effect
	poly(I:C)	0.06(-0.01,0.22)	0.07(-0.01,0.19)	Indirect effect(ACME)
		0.55(-0.03,1.31)	0.57(-0.05,1.29)	Direct effect(ADE)
		0.62(0.03,1.39)	0.64(0.02,1.36)	Total Effect

Table 2. Mediation effect of IL-23 on asthma and exacerbations.

Endpoint	17q variants	Innate ligand	Estimate (95%)	Term
Exacerbations	***rs7216389***	**CpG**	**0.10(0.01, 0.32)**	Indirect effect(ACME)
		poly(I:C)	0.01(-0.02, 0.07)	Indirect effect(ACME)
	rs2305480	**CpG**	**0.08(0.003, 0.34)**	Indirect effect(ACME)
		poly(I:C)	0(-0.03, 0.06)	Indirect effect(ACME)
Asthma	***rs7216389***	**CpG**	**-2.28(-13.1, -0.2)**	Indirect effect(ACME)
		poly(I:C)	-1.05(-7.05, 0.6)	Indirect

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				effect(ACME)
	rs2305480	**CpG**	**-3.07(-17.59, -0.34)**	Indirect effect(ACME)
		poly(I:C)	-1.18(-8.08, 0.25)	Indirect effect(ACME)

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Figure legends

Figure 1. Total effect of risk SNPs on asthma, number of exacerbations, and lung function until age 7, and number of lower respiratory tract infections until 3 years of age.

SNP variants are designated by the names of their associated genes. The association of SNPs and clinical endpoints were examined by regression analysis, where z/t represents test statistics of the coefficients for logistic regression or linear regression. Significant associations ($p < 0.05$) are labeled with an asterisk.

Abbreviations: LRTI: Number of lower respiratory infections until 3 years of age. FEV1: A minimum of three tests with a within-test difference in forced expiratory volume in the first second. PD: The provocative dose of methacholine producing a 20% fall in FEV1. MMEF: maximal mid-expiratory flow. sRaw: A minimum of two assessments of specific airway resistance.

Figure 2. Associations between risk SNPs, clinical endpoints and immune profiles summarized by a principal component analysis.

Immune cytokine profiles were generated based on stimulation of PBMCs from 6-month-old infants with six different ligands activating different arm of the innate immune system. **A**) The association between immune profiles and the clinically associated genomic SNPs as examined by linear regression analysis. SNP variants are designated by the names of their associated genes. **B**) Associations between immune profiles and clinical endpoints as examined by regression models. All significant associations are labeled with an asterisk. z/t represents test statistics of the coefficients for logistic regression or linear regression.

Abbreviations: LRTI: Number of lower respiratory infections until 3 years of age. FEV1: A minimum of three tests with a within-test difference in forced expiratory volume in the first second. PD: The provocative dose of methacholine producing a 20% fall in FEV1. MMEF: maximal mid-expiratory flow. sRaw: A minimum of two assessments of specific airway resistance.

Figure 3. The significant mediation effect transmitted by innate immune responses to CpG and poly(I:C).

The average indirect effect (ADE), the direct effect and the total effect (ACME) from raw and confounder adjusted mediation analysis. The confounding variables adjusted for are: delivery

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mode (Cesarean), older sibling at birth, smoking during 3rd trimester, and antibiotic usage during 3rd trimester.

Figure 4. Composition of the innate immune response mediating the effect of the risk SNPs on asthma endpoints.

Loading plots of the PCA components (PC1 vs. PC3) showing cytokine responses from the CpG and poly(I:C) found to significantly mediate the effect of the 17q21 risk SNPs on asthma and number of asthma exacerbations.

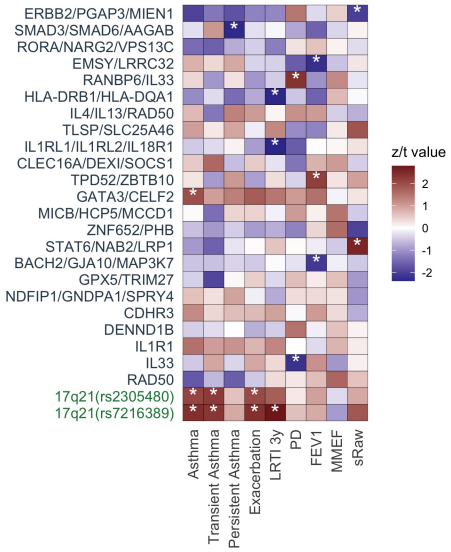
Figure 5. The significant mediation effect transmitted by IL-23 to CpG and poly(I:C).

The average indirect effect (ADE), the direct effect and the total effect (ACME) from mediation analysis.

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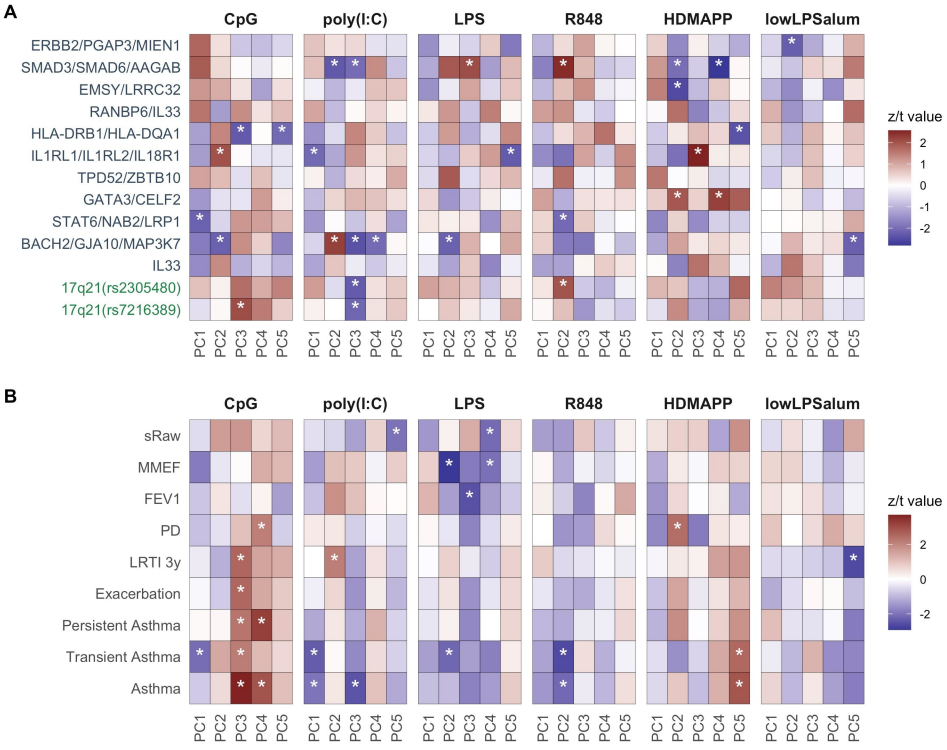
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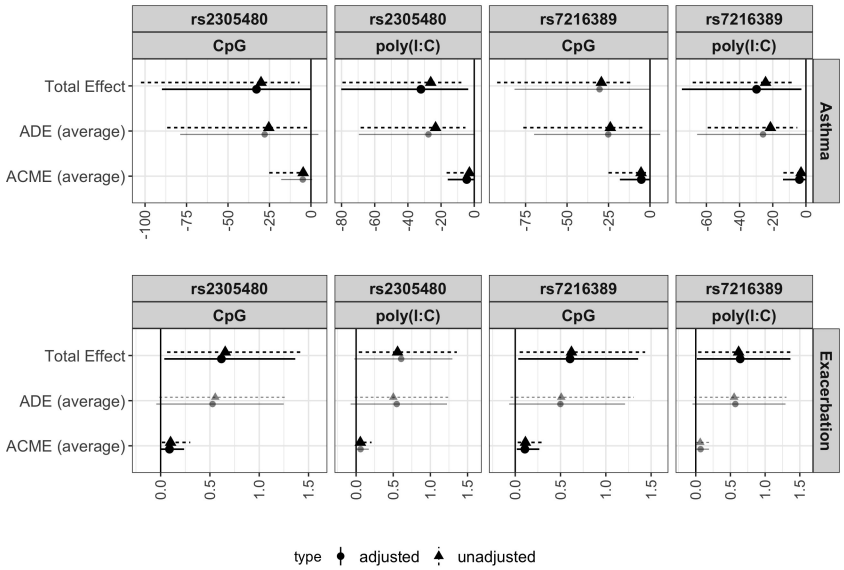
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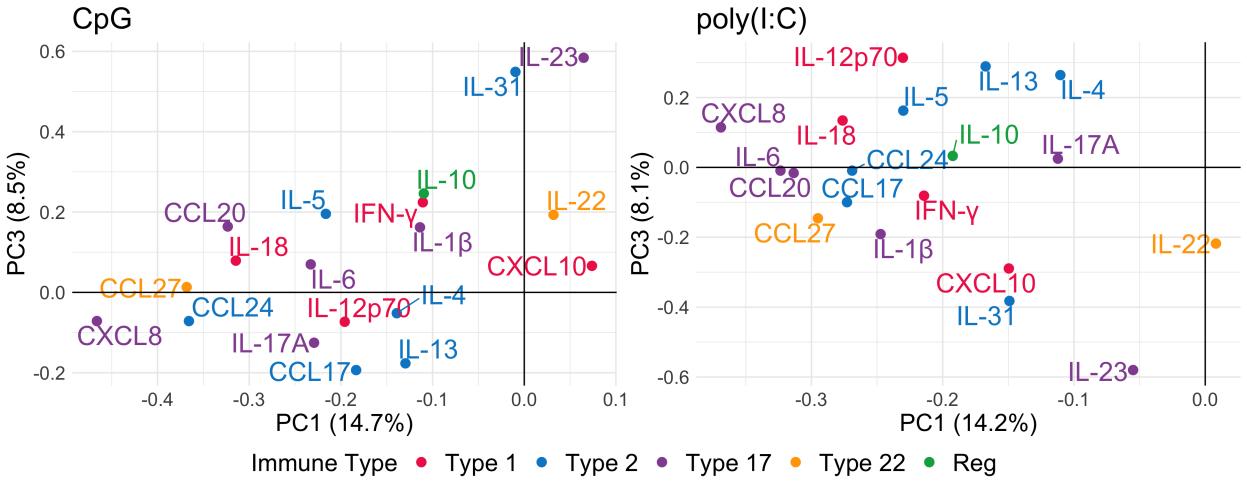
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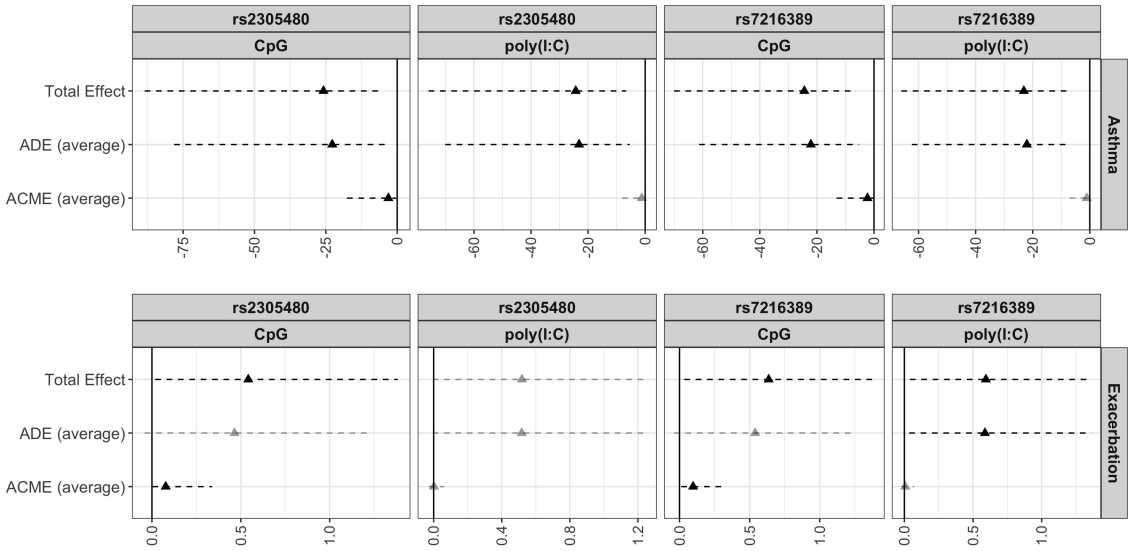
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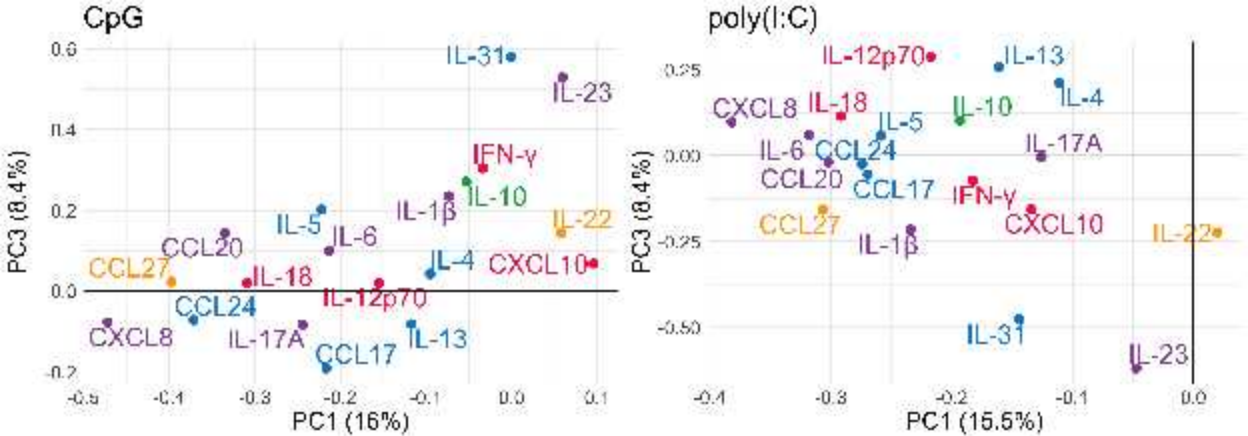
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Socio-economic status
antibiotic use
delivery (Cesarean)
sex
smoking (Yes)
passive smoking (Yes)
antibiotic usage (Yes)
farmer dermatitis
father asthma
mother INF allergy
mother dermatitis
Sex (Boy)
asthma
eczema
CM3
PM 2.5
PM10
SO2
p-value


CpG
poly(I:C)
PC3 (8.4%)
PC1 (16%)
PC1 (15.5%)
IL-31
IL-23
IFN-γ
IL-10
IL-1β
IL-22
IL-5
IL-6
CCL20
CCL27
IL-18
IL-4
CXCL10
IL-12p70
CCL24
CXCL8
IL-17A
CCL17
IL-13
IL-12p70
IL-13
CXCL8
IL-18
IL-10
IL-4
IL-5
IL-6
CCL24
IL-17A
CCL20
CCL17
IFN-γ
CCL27
CXCL10
IL-22
IL-1β
IL-31
IL-23
Immune Type
Type 1
Type 2
Type 17
Type 22
Reg

Online Figure legend:

Figure E1. Associations between covariates and SNPs, clinical endpoints and immune profiles to find possible confounders.

The associations were examined by corresponding regression models and significant associations were labeled with an asterisk. Social circumstances describe the composite score based on mother's highest education level, mother's age at birth, and the annual family income. Abbreviation: mother INH allergy: allergy in the mother to inhalant allergens.

Figure E2. PCA loading plots for sensitivity analysis.

Loading plots of the PCA components found to significantly mediate the effect of the risk SNPs on asthma and number of asthma exacerbations after removing the IL-23 non-responders.

Online Tables:

Table E1. Levels of secreted cytokines from PBMCs of 6 months old children stimulated with the indicated ligands. Median (IQR) in pg/mL is listed. N=336.

	Unstimulated	LPS	CpG	poly(I:C)	R848	HDMAPP	lowLPSalum
Cytokine							
IFN-γ	0.3 (0.1,0.6)	0.4 (0.2,1.4)	0.7 (0.3,1.8)	0.4 (0.1,0.8)	1.6 (0.4,14)	2.9 (1.1,9.0)	0.4 (0.1,1.1)
IL-12p70	0.0 (0.0,0.1)	0.1 (0.0,0.2)	0.0 (0.0,0.1)	0.0 (0.0,0.1)	0.2 (0.1,0.5)	0.0 (0.0,0.1)	0.1 (0.0,0.2)
IL-18	0.2 (0.0,0.3)	0.2 (0.1,0.5)	0.3 (0.2,0.5)	0.3 (0.2, 0.4)	0.5 (0.2, 1.0)	0.2 (0.1,0.4)	0.2 (0.1, 0.4)
CXCL10	0.5 (0.3,1.3)	0.5 (0.2,1.1)	18.9 (3.6,85)	1.7 (0.5,8.3)	11.8 (3.6,39.8)	4.0 (0.8,17.7)	0.4 (0.2,0.8)
IL-4	0 (0,0)	0 (0,0.1)	0 (0,0)	0 (0,0)	0.1 (0,0.2)	0 (0,0)	0 (0,0.1)
IL-5	0 (0,0)	0 (0,0.1)	0.1 (0,0.2)	0 (0,0.1)	0 (0,0.1)	0 (0,0.1)	0 (0,0.1)
IL-13	0 (0,0.2)	0 (0,0.8)	0 (0,0.5)	0 (0,0.3)	0.5 (0, 1.3)	0 (0,0.4)	0 (0,0.9)

IL-31	0 (0,0.1)	0 (0,0.3)	0 (0,0.2)	0 (0,0.1)	0 (0,0.1)	0 (0,0.1)	0 (0,0.1)
CCL17	3.4 (2.3,5.3)	4.8 (3.2,7.6)	2.7 (1.8,3.9)	4.3 (2.9,6.0)	7.4 (4.4,12.5)	3.9 (2.6,5.8)	4.4 (3.0,6.8)
CCL24	51.9 (15.6,441.0)	162.0 (32.7,424.0)	19.2 (11.2,30.8)	69.4 (13.7,302)	42.8 (18.7,89)	105.0 (16.5,612.0)	113.0 (21.8,370)
IL-1β	0.2 (0,0.6)	2.4 (0.7,11.0)	0.2 (0,0.5)	0.2 (0.1,0.6)	6.6 (1.7,22.2)	0.2 (0.1,0.7)	2.1 (0.5,8.9)
IL-6	0.7 (0.3,1.3)	18.8 (3.0,130.0)	8.9 (4.1,16.6)	1.2 (0.6,2.6)	61.2 (18.2,224.0)	0.8 (0.4,1.7)	13.3 (2.2,106.0)
IL-17A	0.2 (0,0.6)	0.4 (0.1,0.8)	0.2 (0,0.4)	0.2 (0,0.5)	0.4 (0.1,0.7)	0.3 (0,0.8)	0.4 (0.1,0.7)
IL-23	0 (0,0)	0.4 (0,3.3)	0.1 (0,0.6)	0 (0,0.5)	0 (0,1.7)	0 (0,0)	0 (0,2.0)
CCL20	0.3 (0,0.6)	3.8 (0.7,28.9)	0.5 (0.3,0.9)	0.6 (0.2,1.1)	4.7 (1.1,19.8)	0.4 (0.1,0.8)	2.6 (0.6,21.9)
CXCL8	115.0 (44.4,287.0)	552.0 (136.0,4142.0)	128.0 (70.5,278.0)	160.0 (68.9,364.0)	1222.0 (320.0,5333.0)	148 (62.7,445.0)	435.0 (127.0,3784.0)
IL-22	0 (0,0)	0 (0,8.4)	0 (0,5.2)	0 (0,6.2)	5.6 (0,18.9)	0 (0,0)	0 (0,6)
CCL27	11.6 (7.4,17,4)	13.7 (7.8,17.5)	10.0 (6.3,13.9)	10.2 (5.7,16)	11.4 (6.9,15.1)	14.8 (9.8,21.2)	12.5 (8.2,17.1)
IL-10	0.1 (0,0.1)	0.2 (0,1.3)	0.7 (0.2,1.9)	0.1 (0,0.2)	1.8 (0.5,6)	0.1 (0,0.2)	0.1 (0,0.1)

Table E2. Baseline characteristics of covariates and clinical endpoints.

Covariates	$COPSAC_{2000}$ N=411	Study Cohort N=336	Excluded children N=75	P value
Antibiotics in 3rd trimester	14.4% (59)	13.4%(45)	18.7%(14)	0.32
Cat in house in pregnancy	15.2% (60)	14.2%(47)	19.7%(13)	0.35
Delivery mode (Cesarean)	21.2% (87)	21.1%(71)	21.3%(16)	1.00
Paternal asthma*	14.2% (57)	13.8%(45)	16.2%(12)	0.72
Paternal dermatitis*	10.7% (36)	11.7%(33)	5.5%(3)	0.26
Maternal dermatitis*	42.6% (147)	42.2%(122)	44.6%(25)	0.85
Maternal allergy to inhaled allergen*	86.9% (357)	87.8%(295)	82.7%(62)	0.32
Any older siblings	40.4% (166)	39.3%(132)	45.3%(34)	0.40
Passive smoke in 3rd trimester	10.0% (41)	9.5%(32)	12.0%(9)	0.66
Sex(Boy)	49.4% (203)	50.3%(169)	445.3%(34)	0.52
Smoking in 3rd trimester	15.3% (63)	14.3%(48)	20.0%(15)	0.29
Household income above 800K DKK	8.7% (33)	8.8%(29)	7.7%(4)	1.00
Mother's age at birth (yr), mean(SD)	30.1 (4.5)	30.1(4.5)	29.6(4.4)	0.35^{t}
Mother's highest education**	15.2% (58)	15.2%(50)	15.4%(8)	1.00
Asthma until 7y*	17.5%(72)	18.5%(62)	13.3%(10)	0.38
Transient asthma*	9.3% (27)	9.7%(25)	6.5%(2)	0.80
Persistent asthma*	15.2%(47)	14.4%(39)	21.6%(8)	0.36
Exacerbation*	20.4%(64)	19.1%(53)	31.4%(11)	0.14
LRTI before 3y*	56.3%(188)	55.7%(165)	60%(23)	0.70
FEV1, mean(SD) *	-0.03(0.99)	-0.01(1.0)	-0.23(0.90)	0.20^{t}
MMEF, mean(SD) *	-0.11(1.04)	-0.11(1.04)	-0.06(1.07)	0.80^{t}
sRaw, mean(SD) *	0.11(1.01)	0.11(1.03)	0.18(0.88)	0.67^{t}
PD, mean(SD) *	-0.09(1.03)	-0.05(1.04)	-0.36(0.87)	0.10^{t}

Baseline comparisons between children in the study cohort and excluded children. Except Household income above 800K DKK, which was compared by unpaired student's t-test, all the other comparisons were done by Pearson's chi-squared test. t: unpaired student's t-test. *Doctor diagnosed disease. **University education.

Table E3. Frequency of genotypes for children having successful PBMC stimulation.

Genes	rsID/ALT	Value	N	Frequency	Consequence
RAD50	rs6871536/C	0	177	0.60	Intron
		1	101	0.34	
		2	19	0.06	
IL33	rs928413/C	0	153	0.52	Regulatory
		1	124	0.42	
		2	20	0.07	
IL1R1	rs1558641/G	0	4	0.01	Intron
		1	73	0.25	
		2	220	0.74	
DENND1B	rs2786098/T	0	189	0.64	Intron
		1	95	0.32	
		2	13	0.04	
CDHR3	rs6967330/A	0	203	0.68	Missense
		1	80	0.27	
		2	14	0.05	
17q(GSDMB/ORMDL3)	rs7216389/C	0	230	0.70	Intron
		1	101	0.30	
17q(GSDMB/ORMDL3)	rs2305480/G	0	196	0.66	Missense
		1	101	0.34	
NDFIP1/GNDPA1/SPRY4	rs7705042/C	0	144	0.49	Intron
		1	120	0.40	

		2	33	0.11	
GPX5/TRIM27	rs1233578/A	0	5	0.02	Intergenic
		1	76	0.26	
		2	216	0.73	
BACH2/GJA10/MAP3K7	rs2325291/A	0	123	0.41	Intron
		1	137	0.46	
		2	37	0.13	
STAT6/NAB2/LRP1	rs167769/C	0	52	0.18	Intron
		1	142	0.48	
		2	103	0.35	
ZNF652/PHB	rs17637472/G	0	57	0.19	Intron
		1	145	0.49	
		2	95	0.32	
MICB/HCP5/MCCD1	rs2855812/G	0	24	0.08	Intron
		1	122	0.41	
		2	151	0.51	
GATA3/CELF2	rs2589561/G	0	7	0.02	Intergenic
		1	85	0.29	
		2	205	0.69	
TPD52/ZBTB10	rs12543811/A	0	43	0.15	Intron
		1	124	0.42	
		2	130	0.44	
CLEC16A/DEXI/SOCS1	rs17806299/A	0	200	0.67	Intron
		1	90	0.30	

		2	7	0.02	
IL1RL1/IL1RL2/IL18R1	rs1420101/C	0	49	0.17	Intron
		1	142	0.48	
		2	106	0.36	
TLSP/SLC25A46	rs10455025/A	0	47	0.16	Intergenic
		1	131	0.44	
		2	119	0.04	
IL4/IL13/RAD50	rs20541/G	0	14	0.05	Missense
		1	107	0.36	
		2	176	0.59	
HLA-DRB1/HLA-DQA1	rs9272346/G	0	120	0.40	Intron
		1	132	0.45	
		2	45	0.15	
RANBP6/IL33	rs992969/G	0	20	0.07	Intergenic
		1	121	0.41	
		2	156	0.53	
EMSY/LRRC32	rs7927894/C	0	48	0.16	Intergenic
		1	148	0.50	
		2	101	0.34	
RORA/NARG2/VPS13C	rs11071558/G	0	227	0.77	Intron
		1	66	0.22	
		2	4	0.01	

SMAD3/SMAD6/AAGAB	rs2033784/A	0	33	0.11	Intron
		1	99	0.33	
		2	165	0.56	
ERBB2/PGAP3/MIEN1	rs2952156/G	0	33	0.11	Intron
		1	121	0.41	
		2	143	0.48	

Table E4: Mediation effect of immune patterns on the relationship of combined 17q risk and Asthma/Exacerbation

Endpoint	17q variants	Stimuli	Estimate(95%CI)	Term
Exacerbation	17q combined risk	CpG	0.01(0.00, 0.03)	Indirect effect(ACME)
		poly(I:C)	0.01(0.0, 0.03)	Indirect effect(ACME)
Asthma	17q combined risk	CpG	-1.27(-11.00, 0.66)	Indirect effect(ACME)
		poly(I:C)	**-1.17(-12.30, -0.10)**	Indirect effect(ACME)

Table E5. Associations between selected cytokines and clinical outcomes

		Asthma		**Exacerbatiom**	
Innate ligand	cytokine	HR	P	IRR	P
CpG	**IL-23**	**1.09**	**0.02**	**1.13**	**0.02**
	IL-31	1.06	0.17	1.05	0.45
poly(I:C)	**IL-23**	**1.08**	**0.03**	1.02	0.65
	IL-31	1.05	0.26	1.07	0.28

Table E6. Levels of secreted cytokines from PBMCs of 6 months old children after removing IL-23 non-responders stimulated with the indicated ligands. Median (IQR) in pg/mL is listed. N=266

	Control	LPS	CpG	poly(I:C)	R848	HDMAPP	lowLPSalum
Cytokine							
IFN-γ	0.3 (0.1,0.6)	0.5 (0.2,2.1)	0.8 (0.4,2)	0.4 (0.1,0.8)	3.4 (0.5,23.7)	3.4 (1.2,12)	0.5 (0.1,1.6)
IL-12p70	0 (0,0.1)	0.1 (0,0.2)	0 (0,0.1)	0 (0,0.1)	0.2 (0.1,0.6)	0 (0,0.1)	0.1 (0,0.2)
IL-18	0.2 (0,0.3)	0.3 (0.1,0.5)	0.3 (0.2,0.5)	0.3 (0.2,0.4)	0.5 (0.3,1.1)	0.2 (0.1,0.4)	0.3 (0.1,0.4)

CXCL10	0.6 (0.3,1.3)	0.5 (0.2,1.1)	26.4 (5.1,104.3)	2.1 (0.6,9.1)	13 (4.7,47.2)	6 (0.9,21.5)	0.4 (0.2,0.8)
IL-4	0 (0,0)	0 (0,0.2)	0 (0,0)	0 (0,0)	0.1 (0,0.3)	0 (0,0)	0 (0,0.1)
IL-5	0 (0,0.1)	0 (0,0.2)	0.1 (0,0.2)	0 (0,0.1)	0 (0,0.1)	0 (0,0.1)	0 (0,0.1)
IL-13	0 (0,0.3)	0.3 (0,0.9)	0 (0,0.5)	0 (0,0.3)	0.7 (0,1.6)	0 (0,0.5)	0.2 (0,1.1)
IL-31	0 (0,0.2)	0 (0,0.3)	0 (0,0.2)	0 (0,0.2)	0 (0,0.1)	0 (0,0.1)	0 (0,0.1)
CCL17	3.5 (2.3,5.4)	5.3 (3.3,8)	2.7 (1.9,4)	4.3 (2.9,6.2)	7.9 (4.7,13.2)	4 (2.7,6.2)	4.6 (3.2,7.1)
CCL24	88.3 (16.8,589.2)	230.5 (49.6,481.5)	20.1 (11.3,33.8)	99.6 (15.6,403)	45.4 (20.5,98)	172.6 (23.3,893.8)	204.7 (31.9,444.5)
IL-1β	0.2 (0.1,0.6)	3.3 (1,14.6)	0.2 (0.1,0.6)	0.2 (0.1,0.7)	8.9 (2.2,25.4)	0.2 (0.1,0.8)	2.8 (0.8,10.9)
IL-6	0.8 (0.4,1.4)	27.9 (3.3,182.1)	10.1 (4.6,18.2)	1.4 (0.7,2.7)	96 (19.5,284.3)	0.9 (0.4,2)	17.9 (2.9,145.9)
IL-17A	0.3 (0,0.7)	0.5 (0.1,1)	0.2 (0,0.4)	0.3 (0,0.6)	0.4 (0.1,0.8)	0.4 (0.1,1)	0.4 (0.1,0.8)
IL-23	0 (0,0.1)	1.2 (0,4.4)	0.4 (0,0.8)	0.1 (0,0.6)	0.5 (0,2.6)	0 (0,0)	0.2 (0,3.5)
CCL20	0.3 (0.1,0.6)	6.3 (0.8,38.6)	0.6 (0.3,1)	0.6 (0.3,1.1)	8.7 (1.5,25.1)	0.4 (0.2,0.9)	4.8 (0.6,28.8)
CXCL8	133.4 (55.3,330.9)	845.3 (176,5882.1)	149.3 (75.8,312.9)	182.4 (72.5,448.4)	1854.8 (379.6,6930.5)	192 (68.1,565.3)	619.3 (150.5,5175.9)
IL-22	0 (0,0)	0 (0,9.5)	0 (0,6.9)	0 (0,7.1)	10 (0,23.4)	0 (0,0)	0 (0,8.3)
CCL27	12.2 (7.4,18.3)	13.8 (7.9,18.2)	9.7 (6.3,13.9)	10.8 (6,17.8)	11.4 (6.7,15.3)	16.2 (10.3,22.7)	12.6 (7.9,17.4)
IL-10	0.1 (0,0.1)	0.3 (0.1,1.8)	1 (0.3,2.3)	0.1 (0,0.2)	2.6 (0.6,6.9)	0.1 (0,0.2)	0.2 (0,1.6)

Table E7: Mediation effect of immune patterns on asthma after removal of IL-23 non-responders

Asthma			
17q variants	Innate ligand/ Immune Pattern	Estimate(95%CI) unadjusted	Term
rs2305480	CpG/PC3	**-4.54(-24.33, -0.69)**	Indirect effect(ACME)
		-33.73(-127.99, -10.39)	Direct effect(ADE)
		-38.28(-148.5, -15.22)	Total Effect
	poly(I:C)/PC3	**-3.17(-26.1, -0.33)**	Indirect effect(ACME)
		-34.11(-131.23, -14.42)	Direct effect(ADE)
		-37.28(-146.73, -16.86)	Total Effect
rs7216389	CpG/PC3	**-5.41(-26.2, -1.51)**	Indirect effect(ACME)
		-24.03(-82.84, -3.4)	Direct effect(ADE)
		-29.45(-96.32, -9.07)	Total Effect
	poly(I:C)/PC3	**-3.04(-21.23, -0.42)**	Indirect effect(ACME)
		-23.77(-87.75, -6.89)	Direct effect(ADE)
		-26.81(-99.91, -9.4)	Total Effect

Table E8: Mediation effect of immune patterns on exacerbation after removal of IL-23 non-responders

Exacerbation			
17q variants	Innate ligand/ Immune Pattern	Estimate(95%CI) unadjusted	Term
rs2305480	CpG/PC3	**0.07(0.01, 0.25)**	Indirect effect(ACME)
		0.65(0.01, 1.56)	Direct effect(ADE)
		0.72(0.08, 1.64)	Total Effect
	poly(I:C)/PC3	**0.05(0, 0.21)**	Indirect effect(ACME)
		0.7(0.04, 1.6)	Direct effect(ADE)
		0.75(0.08, 1.67)	Total Effect
rs7216389	CpG/PC3	**0.10(0.02, 0.28)**	Indirect effect(ACME)
		0.49(-0.12, 1.45)	Direct effect(ADE)
		0.59(-0.03, 1.57)	Total Effect
	poly(I:C)/PC3	**0.07(0, 0.22)**	Indirect effect(ACME)
		0.57(-0.11, 1.44)	Direct effect(ADE)
		0.63(-0.04, 1.56)	Total Effect

Table E9: Mediation effect of IL-23 on asthma and exacerbation after removal of IL-23 non-responders

Endpoint	17q variants	Stimuli	Estimate(95%CI)	Term
Exacerbation	rs2305480	CpG	**0.12(0.01, 0.54)**	Indirect effect(ACME)
		poly(I:C)	0(-0.11, 0.03)	Indirect effect(ACME)
	rs7216389	CpG	**0.12(0.02, 0.48)**	Indirect effect(ACME)
		poly(I:C)	0.01(-0.03, 0.1)	Indirect effect(ACME)
Asthma	rs2305480	CpG	**-5.21(-43.86, -1.26)**	Indirect effect(ACME)
		poly(I:C)	-1.73(-13.73, 0.39)	Indirect effect(ACME)
	rs7216389	CpG	**-2.94(-21.29, -0.37)**	Indirect effect(ACME)
		polyIC	-1.25(-10.5,0.92)	Indirect effect(ACME)

Online method

Stimulation of PBMCs

PBMCs were isolated by density centrifugation of blood samples collected at 6 months of age and stored for up to 12 years at -140oC. After thawing, the cells were stimulated in sterile U-bottomed 96-well plates at 5x105 cells/well; 200 µL total volume/well in complete media (RPMI 1640 medium completed with 2mM L-glutamine (Cambrex, East Rutherford, NJ), 0.1 M HEPES (Lonza), 100 U/ml penicillin/streptomycin (Lonza) and 10% heat-inactivated fetal bovine serum) for 40 hours at 37oC and 5% CO2 in a humidified incubator with addition of one of 6 ligands activating different innate immune pathways (lipopolysaccharide (ultrapure E. coli LPS (tlrl-pelps, Invivogen, France), 200 ng/mL)), unmethylated cytosine poly guanosine DNA (CpG (ODN2216, TIB MOLBIOL, Berlin), 30 µg/mL), polyinosinic:polycytidylic acid (poly(I:C) (tlrl-picw-250, Invivogen, France), 50 µg/mL), resiquimod (R848 (tlrl-r848-250, Invivogen, France), 2 µg/mL), 1-hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP (I-M055, Echelon Biosciences, USA), 0.1 µg/mL) and aluminum potassium sulfate (alum crystals (tlrl-alk, Invivogen, France), 100 µg/mL) in concert with low dose LPS (5 ng/mL) (lowLPSalum) or complete media alone (unstimulated). Supernatants were harvested and stored at -80oC until the quantification of the following 18 cytokines and chemokines: IFN-γ, IL-12p70, IL-18, IL-4, IL-5, IL-13, IL-31, CCL17, CCL24, IL-1β, IL-6, IL-17A, IL-23, CCL20, CXCL8, IL-22, CCL27 and IL-10 levels by customized multiplex immunoassays from MesoScale Discovery read on a Sector Imager 6000 (MSD, Gaithersburg, MD, USA).

Asthma risk loci

Based on a recent meta-GWAS and other GWAS studies [1]–[3], we selected 25 top risk SNPs for asthma encompassing two variants in the 17q21 locus and *RAD50, IL33, IL1R1, DENND1B, CDHR3, GPX5/TRIM27, NDFIP1/GNDPA1/SPRY4, BACH2/GJA10/MAP3K7, STAT6/NAB2/LRP1, ZNF652/PHB, MICB/HCP5/MCCD1, GATA3/CELF2, TPD52/ZBTB10, CLEC16A/DEXI/SOCS1, IL1RL1/IL1RL2/IL18R1, TSLP/SLC25A46, IL4/IL13/RAD50, HLA-DRB1/HLA-DQA1, RANBP6/IL33, EMSY/LRRC32, RORA/NARG2/VPS13C,*

SMAD3/SMAD6/AAGAB, ERBB2/PGAP3/MIEN1 genes. Allelic discrimination of those SNPs in $COPSAC_{2010}$ was investigated by genome-wide genotyping using the Infinium HumanOmniExpressExome BeadChip Kit (Illumina, San Diego, Calif). We include SNPs with call rate > 95%, minor allele frequency (MAF) >1% , Hardy-Weinberg equilibrium P value $>1 \times 10^{-6}$ as well as imputation quality score >0.5 if the SNPs are from imputation.[3]

Clinical endpoints

Asthma was diagnosed based on a predefined and validated diagnostic algorithm[4], requiring all of the following: (1) recurrent wheeze, i.e. diary recordings of at least 5 episodes of troublesome lung symptoms within the preceding 6 months, each lasting at least 3 days; (2) asthmatic symptoms, e.g. exercise-induced symptoms, prolonged nocturnal cough, persistent cough outside common cold; (3) rescue use of inhaled short-acting bronchodilator; and (4) response to a 3-month trial of inhaled corticosteroids and relapse after end of treatment. Persistent asthma refers to children fulfilling the above diagnostic criteria at any time point during childhood and still needing inhaled corticosteroids to control their symptoms in the 7^{th} year of life, whereas transient asthma denotes children fulfilling the diagnostic criteria, but outgrowing their symptoms before age 7.

Asthma exacerbations were defined by symptoms requiring hospitalization, oral prednisolone (1-2 mg/kg for 3 to 7 days) or high-dose ICS treatment (at least 1600 mcg budesonide per day for 14 days).[5] If the child was admitted directly to the hospital without involvement of the COPSAC research unit, hospital records were retrieved and reviewed to confirm that symptom history and treatment fulfilled the above criteria.

Lung function at age 7 included: (a) spirometry using a MasterScope Pneumoscreen spirometer (Erich Jäeger, Würzburg, Germany). A minimum of three tests with a within-test difference in forced expiratory volume in the first second (FEV1) of maximum 10% was completed. The best

FEV1 and the corresponding maximal mid-expiratory flow (MMEF) were used in the analysis after calibrating for age, sex and height; (b) whole-body plethysmography using a MasterScope sealed bodybox (Erich Jäeger, Würzburg, Germany). A minimum of two assessments of specific airway resistance (sRaw) with a within-test difference of maximum 0.3 kPa/s were obtained, using the mean of the two measurements in the analysis after calibrating the values for age, sex and height; and (c) bronchial responsiveness by measuring FEV1 after a saline inhalation and after subsequent inhalations of methacholine in quadrupling dose steps (APS Pro, CareFusion, 234 GmbH, Germany).[6] The provocative dose of methacholine producing a 20% fall in FEV1 (PD20) was estimated from the dose-response curve fitted with a logistic function.

Lower respiratory tract infections (LRTI) at age 0-3 years included pneumonia and bronchiolitis. Pneumonia was defined by significant cough, tachypnea, fever, and abnormal lung stethoscopy, whereas bronchiolitis was diagnosed in children with cough, tachypnea, chest retractions, auscultative widespread crepitation and/or rhonchi before age 1 year.[7]–[9]

Statistical analysis

Data transformation

The concentration of cytokines and chemokines in response to the innate ligand stimulations were adjusted for background levels by dividing the concentration of the stimulated cells with that of unstimulated PBMCs for each child. Thereafter, the quotient was log-transformed to improve normality before the statistical analysis. To consider the synergistic effects among the cytokines, a PCA for each ligand was employed to decompose the 18-dimensional cytokine data structure into fewer components utilizing the first five PCs in the following analyses.

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