ORIGINAL ARTICLE

Immune Responsiveness to LPS Determines Risk of Childhood Wheeze and Asthma in 17q21 Risk Allele Carriers

Sabina Illi[1,2], Martin Depner[1], Petra Ina Pfefferle[2,3], Harald Renz[2,4,5], Caroline Roduit[6,7,8], Diana Hazard Taft[9], Karen M. Kalanetra[9], David A. Mills[9], Freda M. Farquharson[10], Petra Louis[10], Elisabeth Schmausser-Hechfellner[1], Amandine Divaret-Chauveau[11,12,13], Roger Lauener[6,8], Anne M. Karvonen[14], Juha Pekkanen[14,15], Pirkka V. Kirjavainen[14,16], Marjut Roponen[17], Josef Riedler[18], Michael Kabesch[19], Bianca Schaub[2,20], Erika von Mutius[1,2,20], and the PASTURE Study Group

[1]Institute of Asthma and Allergy Prevention, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany; [2]German Center for Lung Research, Germany; [3]Comprehensive Biobank Marburg and [4]Institute of Laboratory Medicine, Philipps University of Marburg, Marburg, Germany; [5]Department of Clinical Immunology and Allergology, Laboratory of Immunopathology, Sechenov University, Moscow, Russia; [6]Christine Kühne Center for Allergy Research and Education, Davos, Switzerland; [7]Children's Hospital, University of Zürich, Zürich, Switzerland; [8]Children's Hospital of Eastern Switzerland, St. Gallen, Switzerland; [9]Department of Food Science and Technology, University of California, Davis, California; [10]The Rowett Institute, University of Aberdeen, Foresterhill, Aberdeen, United Kingdom; [11]UMR 6249 Chrono-environment, Centre National de la Recherche Scientifique and University of Franche-Comté, Besançon, France; [12]EA3450 Development, Adaptation and Handicap, University of Lorraine, Nancy, France; [13]Pediatric Allergy Department, University Hospital of Nancy, Nancy, France; [14]Department of Health Security, Finnish Institute for Health and Welfare, Kuopio, Finland; [15]Department of Public Health, University of Helsinki, Helsinki, Finland; [16]Institute of Public Health and Clinical Nutrition and [17]Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio, Finland; [18]Children's Hospital Schwarzach, Schwarzach, Austria; [19]Department of Pediatric Pneumology and Allergy, University Children's Hospital Regensburg at the Hospital St. Hedwig of the Order of St. John, University of Regensburg, Regensburg, Germany; and [20]Dr. von Hauner Children's Hospital, Ludwig Maximilians University Munich, Munich, Germany

ORCID IDs: 0000-0003-4994-7732 (S.I.); 0000-0003-2115-2399 (P.L.); 0000-0003-2257-2934 (A.M.K.); 0000-0002-1083-8777 (J.P.); 0000-0003-1652-8873 (B.S.); 0000-0002-8893-4515 (E.v.M.).

Abstract

Rationale: In murine models, microbial exposures induce protection from experimental allergic asthma through innate immunity.

Objectives: Our aim was to assess the association of early life innate immunity with the development of asthma in children at risk.

Methods: In the PASTURE farm birth cohort, innate T-helper cell type 2 (Th2), Th1, and Th17 cytokine expression at age 1 year was measured after stimulation of peripheral blood mononuclear cells with LPS in $n = 445$ children. Children at risk of asthma were defined based on single-nucleotide polymorphisms at the 17q21 asthma gene locus. Specifically, we used the SNP rs7216389 in the *GSDMB* gene. Wheeze in the first year of life was assessed by weekly diaries and asthma by questionnaire at age 6 years.

Measurements and Main Results: Not all cytokines were detectable in all children after LPS stimulation. When classifying detectability of cytokines by latent class analysis, carrying the 17q21 risk allele rs7216389 was associated with risk of wheeze only in the class with the lowest level of LPS-induced activation: odds ratio (OR), 1.89; 95% confidence interval [CI], 1.13–3.16; $P = 0.015$. In contrast, in children with high cytokine activation after LPS stimulation, no association of the 17q21 risk allele with wheeze (OR, 0.63; 95% CI, 0.29–1.40; $P = 0.258$, $P = 0.034$ for interaction) or school-age asthma was observed. In these children, consumption of unprocessed cow's milk was associated with higher cytokine activation (OR, 3.37; 95% CI, 1.56–7.30; $P = 0.002$), which was in part mediated by the gut microbiome.

Conclusions: These findings suggest that within the 17q21 genotype, asthma risk can be mitigated by activated immune responses after innate stimulation, which is partly mediated by a gut–immune axis.

Keywords: innate immune response in children; 17q21 genotype; farm environment; unprocessed cow's milk; gut microbiome

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A complete list of the PASTURE Study Group members may be found before the beginning of the References.

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At a Glance Commentary

Scientific Knowledge on the Subject: Asthma risk in children is strongly conferred by a specific genotype, namely variants at the chromosome 17q21 locus, which may interact with various environmental exposures. On the other hand, animal models have shown that specific microbe-rich environments may provide protection against asthma by engaging and shaping the innate immune response. In line with these findings, growing up on a traditional farm is associated with less wheeze and asthma in children, indicating a crucial role for a microbe-rich environment in early life.

What This Study Adds to the Field: Findings from the rural PASTURE birth cohort indicate that asthma development in 17q21 risk allele carriers is associated with an impaired immune responsiveness to the potent innate stimulus LPS. In contrast, risk allele carriers with an activated T-helper cell type 1 (Th1)/Th2/Th17 immune response after innate stimulation by the age of 1 year were at no increased risk of wheeze and asthma up to the age of 6 years. Oral rather than inhaled exposures and the resulting changes in the compositional structure of the early gut microbiome restored responsiveness to microbial LPS. These findings suggest that within the 17q21 genotype, asthma risk might be mitigated by restoring Th1/Th2/Th17 activation after microbial stimulation.

A multitude of studies has shown that growing up on a traditional farm is associated with less wheeze and asthma in children, indicating a crucial role for a microbe-rich environment in early life (1–4). The underlying mechanisms are, however, still not yet clear. Animal models have shown that specific microbe-rich environments may provide protection against asthma by engaging and shaping the innate immune response (5).

On the other hand, asthma risk in children is strongly conferred by a specific genotype, namely variants at the chromosome 17q21 locus (6–10). Symptomatic risk allele carriers constitute a phenotype associated with early episodes of viral wheeze (11), repeated exacerbations (12, 13), and an increased risk for persistent wheeze and asthma at school age (14). The 17q21 locus interacts with various environmental exposures and may thus constitute a switch toward risk when interacting with higher number of siblings and passive smoke exposure (14, 15) or toward protection when interacting with furred pets and farm animal sheds (14, 16, 17). The underlying immunomodulatory mechanisms are not yet clear (18).

We hypothesized that increased expression of peripheral blood cytokines after stimulation with a potent innate stimulus, which we used as a marker for an activated early innate immune response, could counteract the increased risk for wheeze and asthma in 17q21 risk allele carriers. Moreover, we hypothesized that a microbe-rich environment in early life shapes a child's early immune response and may thus play a major role in this complex interaction, specifically within the setting of a rural study with traditional farm and nonfarm environments. Some of the results of this study have been previously reported in the form of an abstract (19).

Methods

Study Population

PASTURE (Protection against Allergy—Study in Rural Environments) is a large prospective birth cohort study conducted in rural areas of five European countries: Austria, Finland, France, Germany, and Switzerland. The study design has been described earlier (20). Briefly, pregnant women were recruited during the last trimester of pregnancy between 2002 and 2005. Women living on an actively run farm where livestock was held were considered farming women. Pregnant women living in the same rural area but not occupationally involved in farming activities were assigned to the nonfarm reference group. In all, 1,133 women were included in the study (530 farming and 603 nonfarming women). Study population for the current analyses were all children with available cytokine measurements at 1 year. The study was approved by local research ethics committees in each country, and written informed consent was obtained from the children's parents.

Questionnaires

Extensive questionnaires were administered in the third trimester of pregnancy and repeatedly after birth until 6 years. Furthermore, at age 8–53 weeks, parents were asked to complete weekly and monthly diaries. All questionnaires and diaries assessed illnesses as well as farm-related and

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Correspondence and requests for reprints should be addressed to Sabina Illi, Ph.D., Institute of Asthma and Allergy Prevention, Helmholtz Zentrum München, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany. E-mail: sabina.illi@helmholtz-muenchen.de.

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other environmental exposures. Wheeze in the first year of life was defined as any wheeze during the previous 7 days as registered by the weekly diaries. Asthma was defined as a physician's diagnosis of asthma or recurrent obstructive bronchitis established until 6 years.

Blood Sampling

At birth, cord blood samples were taken for genotyping; venous blood samples were collected at age 1 year.

Cytokine production after stimulation with innate stimulus. Cytokine levels of IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, and TNF-α were measured after stimulation of whole blood with LPS (0.1 μg/ml; kindly provided by Profs. Holst and Brade, Borstel) for 24 hours at age 1 year in all study centers except France. Detection limits of cytokines, percent nondetects, and basic descriptions of cytokine concentrations are given in Table E1 in the online supplement.

Genotyping of SNP at 17q21. Genotyping was performed at the Centre National de Génotypage, Evry, France, using the iPLEX Gold technology, and SNPs at the 17q21 asthma gene locus were genotyped. For the current analyses, we used the SNP rs7216389 in the *GSDMB* (gasdermin-B) gene, which was coded for its risk allele (T). This SNP within the 17q21 locus has been associated with childhood asthma (6, 9–11, 21) and has been used in previous analyses of the PASTURE cohort (14, 22). Of all 939 children with available DNA samples from cord blood, quality of DNA and genotyping was sufficient for 896 children.

Fecal Sampling

Fecal samples were collected from 720 children at 1 year, and DNA was extracted to determine bacterial communities defined by 16S rRNA gene analyses and preprocessed as recently described (23). Microbial variables included in statistical analyses comprised aggregated variables derived from the α-diversity (richness, i.e., the number of amplicon sequence variants, and Shannon index) and principal coordinate analysis (PCoA). Furthermore, estimated microbiome age was calculated, and two butyrate variables were created: a butyrate score based on bacterial taxa predicting butyrate production and a gene assay based on the relative abundance of the gene encoding the main enzyme of bacterial butyrate metabolism (23).

Statistical Analyses

For all analyses on the 17q21-SNP rs7216389, we combined the heterozygous (CT) and homozygous (TT) as "risk allele carriers." For the analysis of dichotomous or categorical variables, we used the χ^2 test.

Because concentrations of cytokines after LPS stimulation were below detection level in a large proportion of children, all cytokine measurements were dichotomized at detection level (Table E1). For the grouping of children according to cytokine profiles, we then conducted a latent class analysis on the dichotomized measurements of cytokines with a detectability rate of less than 99%. As a sensitivity analysis, we conducted the same latent class analysis including all cytokines irrespective of the detectability rate.

To assess the association of the 17q21-SNP, which we used as indicator for an increased asthma risk, with the outcome of wheeze in the first year of life, general estimation equations (GEE) were conducted, stratified for the latent classes of cytokine detectability. Furthermore, multinomial logistic regression models with the latent cytokine class after LPS stimulation as categorical outcome were performed in risk allele carriers, the low cytokine class being used as reference category. All exposures with a *P* value 0.1 or less were then included in a stepwise variable selection procedure. Similarly, the microbial variables as well as relevant single taxa were included in multinomial logistic regression models.

In addition, we conducted a mediation analysis to assess whether the effect of the relevant environmental exposures on high cytokine class were mediated by the fecal microbiome in the 17q21 risk allele population. All above models were adjusted for study center, GEE models with repeated (weekly) outcomes of wheeze in the first year of life were additionally adjusted for age in weeks, and the final stepwise and mediation models were adjusted for farming and study center. Effect estimates are presented as adjusted odds ratios (ORs), and a *P* value of 0.05 was considered significant. In the mediation analysis, all effects are given as farm- and center-adjusted regression parameters (β). Owing to the exploratory character of the analyses, corrections for multiple testing were not performed. Statistical analyses were performed with SAS 9.4 (the SAS Institute), Mplus 8.1 (Muthén and Muthén), and R 3.41 (www.r-project.org).

Results

Study Population

In total, 445 children had measurements of all assessed cytokines after LPS stimulation at 1 year (Figure E1). The children with LPS-stimulated cytokines and the total PASTURE study population did not differ significantly with respect to relevant exposures or confounders (farming status, gender, parental atopy, older siblings, maternal smoking in pregnancy, maternal education, early farm milk consumption, wheeze, or asthma) (Table E2). Of all 445 children, 377 had information on the 17q21 risk allele, with 257 (68.2%) being a risk allele carrier (185 heterozygous and 72 homozygous).

Cytokine Responsiveness and Latent Classes of Detectable Cytokines after LPS Stimulation

LPS stimulation of peripheral blood mononuclear cells yielded many undetectable measurements of assessed cytokines (Figure 1): although IL-1β, IL-6, IL-10, and TNF-α were detectable in all or nearly all children, a different pattern was observed for the other cytokines, with detectability varying between 3.1% for IL-5 and 80.4% for IFN-γ.

To classify children into distinct patterns of cytokine detectability after the innate stimulus LPS, we dichotomized all cytokines into detectable versus nondetectable and included those with a detectability of less than 99% in a latent class analysis.

The solution yielding best results with respect to objective criteria was a three-class solution (Table 1) yielding patterns of selective responsiveness. The largest latent class ($n = 231$) was comprised of mostly children with a low percentage of detectable IL-4, IL-5, IL-13, IL-17, IL-12p70, and IFN-γ (i.e., of low T-helper cell type 2 [Th2]/low Th1/low Th17 cytokine detectability). For example, IL-12p70 was detectable in 46.5% of the children in total, but only in 1.3% of the children in this class. We thus labeled this class "low class." The "intermediate class" ($n = 119$) showed very high detectability of IL-12p70 and IFN-γ with intermediate detectability of IL-13 and IL-17. In turn, the smallest latent class ($n = 95$) comprised children with high detectability of almost all cytokines after LPS stimulation, which we thus labeled "high class." Of note, in all three resulting classes, IL-1β, IL-6, IL-10, and TNF-α were detectable in all or nearly all children. Inclusion of these cytokines with a

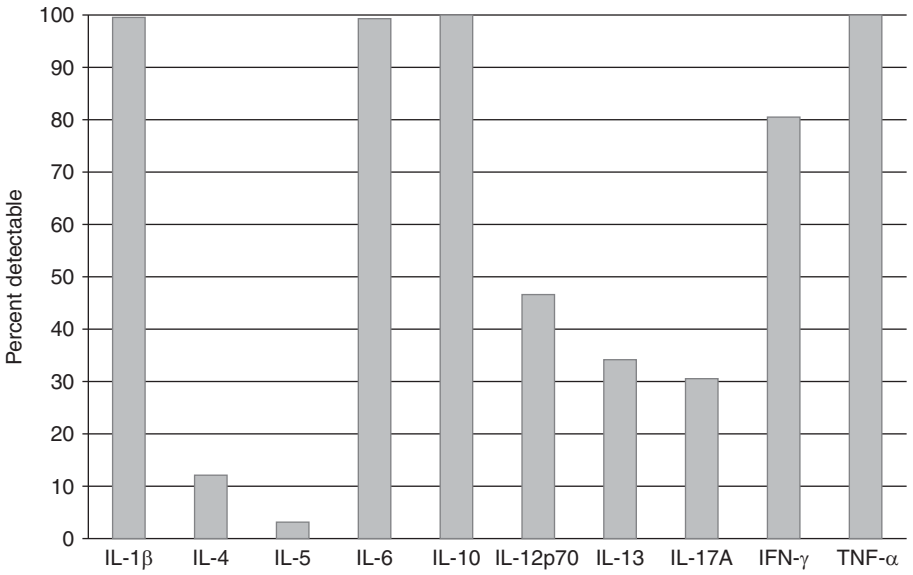

Figure 1. Proportion of samples with detectable LPS-stimulated cytokines at 1 year (percent above detection level); $N = 445$. TNF = tumor necrosis factor.

detectability of 99% or more in the latent class analysis yielded almost identical classes, with only 7 (1.6%) children being assigned to a different class.

17q21 risk allele carriers were similarly distributed over cytokine classes after LPS stimulation (69.0% in low, 65.6% in intermediate, and 69.2% in high cytokine class; $P = 0.620$). Vice versa, cytokine classes after LPS stimulation were similarly distributed in 17q21 risk allele carriers (54.9% low, 24.5% intermediate, and 21.0% high [Table E3]) and noncarriers (52.5%, 27.5%, and 20.0%, respectively; $P = 0.824$).

17q21 Risk Allele Is Associated with More Wheeze in the First Year of Life and More Asthma at 6 Years, but Not in Children with Activated Response

In analyses stratified for the three cytokine classes, we assessed the effect of the 17q21 risk SNP on wheezing in early life using GEEs (Figure 2). In the total population, 17q21 risk allele carriers were at increased risk of having wheezed in the first year, though not significantly so (OR, 1.40; 95% confidence interval [CI], 0.97–2.01; $P = 0.071$). If, however, the child was in the lowest cytokine class, the risk of wheeze in the first year was significantly increased in 17q21 risk allele carriers as compared with children with no risk allele (OR, 1.89; 95% CI, 1.13–3.16; $P = 0.015$). In contrast, in the highest cytokine class, no such effect was observed (OR, 0.63; 95% CI, 0.29–1.40; $P = 0.258$), indicating a significant interaction between 17q21 risk allele and cytokine class on wheeze ($P = 0.034$ for interaction).

Furthermore, children with a 17q21 risk allele were at significantly increased risk of having asthma at 6 years compared with children with no risk allele (OR, 3.66; 95% CI, 1.25–10.72; $P = 0.018$). This genotype-dependent risk was, however, only present in children in the low cytokine class (OR, 3.93; 95% CI, 0.87–17.71; $P = 0.075$), though not significantly so, potentially owing to low numbers (16/124, 12.9% vs. 2/55, 3.6%). In contrast, in the group of children in the high cytokine class, the 17q21 risk allele exerted no effect on the outcome of asthma at 6 years (OR, 1.35; 95% CI, 0.24–7.60; $P = 0.733$).

Table 1. Detectable LPS-stimulated Cytokines at Age 1 Year

			Latent Cytokine Classes							
	Total ($N = 445$)		LC1 (Low Class) [$n = 231$ (51.9%)]		LC2 (Intermediate Class) [$n = 119$ (26.7%)]		LC3 (High Class) [$n = 95$ (21.4%)]		*P* Value	
Cytokines	***n***	**%**	***n***	**%**	***n***	**%**	***n***	**%**	**Chi²**	**Trend**
Included in LCA*										
IL-4	54	12.1	1	0.4	8	6.7	45	47.4	<0.001	<0.001
IL-5	14	3.1	0	0.0	3	2.5	11	11.6	<0.001	<0.001
IL-12p70	207	46.5	3	1.3	112	94.1	92	96.8	<0.001	<0.001
IL-13	152	34.2	23	10.0	34	28.6	95	100.0	<0.001	<0.001
IL-17A	136	30.6	17	7.4	30	25.2	89	93.7	<0.001	<0.001
IFN-γ	358	80.4	145	62.8	118	99.2	95	100.0	<0.001	<0.001
Excluded from LCA†										
IL-1β	443	99.5	231	100.0	118	99.2	94	98.9	0.329	0.154
IL-6	442	99.3	231	100.0	118	99.2	93	97.9	0.104	0.035
IL-10	445	100.0	231	100.0	119	100.0	95	100.0	—	—
TNF-α	445	100.0	231	100.0	119	100.0	95	100.0	—	—

Definition of abbreviations: LCA = latent class analysis; TNF = tumor necrosis factor.
Allocation to latent classes.
*Detectability <99%.
†Detectability ⩾99%.

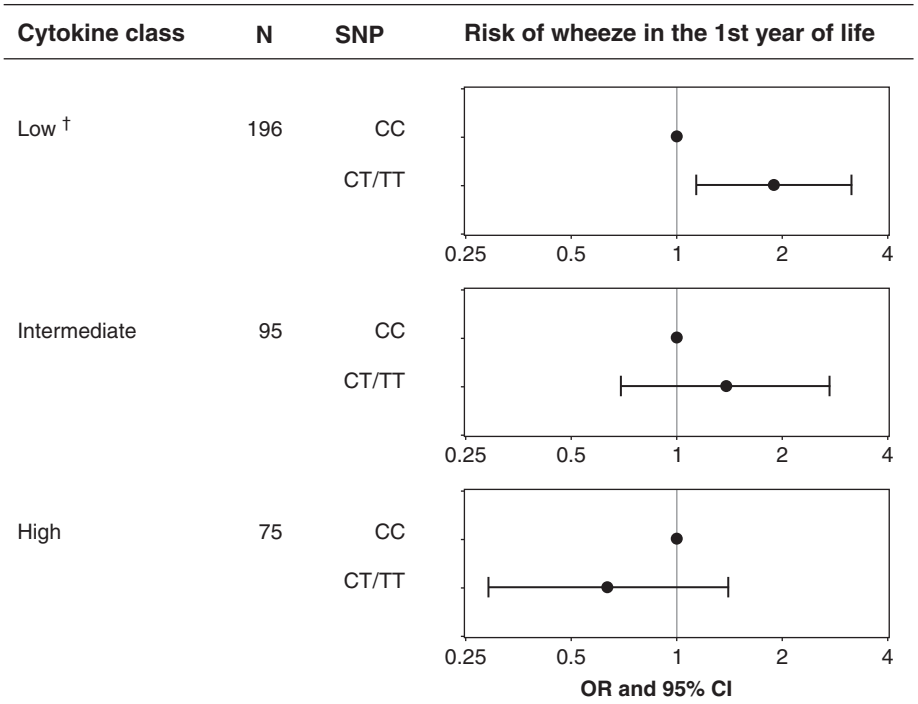

Figure 2. Effect of the 17q21 risk allele on wheeze in the first year of life: Adjusted (for age and center) odds ratios from general estimation equation analyses stratified for cytokine class. CI = confidence interval; OR = odds ratio. †Data shown are adjusted for age only. Owing to small numbers, when adjusting for center, the generalized Hessian matrix is not positive definite, hence no confidence intervals can be calculated. Effect size, however, remains unchanged.

Environmental Exposures Are Associated with High Cytokine Class

As shown above, 17q21 risk allele carriers were only at increased risk of wheeze in the first year of life and of asthma at 6 years if the child was in the lowest cytokine class after LPS stimulation. Thus, the question arose, which factors contribute to a higher cytokine class within the group of 17q21 risk allele carriers and might thus potentially counterbalance the genetically increased risk of wheezing and asthma.

Of all assessed environmental exposures, only farm-related exposures were significantly associated with the high cytokine class in center-adjusted analyses in 17q21 risk allele carriers (Table 2). Similar effects were seen in the total population (Table E4). Among these were exposures both in pregnancy and in the first year of life, such as consumption of farm milk and exposure to stables and farm animals. It is noteworthy that the effect of boiled and unboiled farm milk was of similar magnitude (data not shown). However, consumption of farm milk was not the only diet-related exposure: introduction of a high variety of foods was also associated with the high cytokine class, though not significantly so. Other factors such as sex, birth weight, gestational age, parental atopy, older siblings, maternal smoking, maternal infections in pregnancy, infections in the first year of life defined as the number of weeks with respiratory tract infections, fever or otitis, breastfeeding, regular stay in daycare, and pet keeping in the house showed no significant effect on cytokine class in 17q21 risk allele carriers (data not shown). To disentangle the various factors associated with the high cytokine class, we conducted a stepwise multivariate multinomial logistic regression including all relevant factors from bivariate analyses. The only variable, however, selected into the final model was consumption of farm milk in the first year of life (OR, 3.37; 95% CI, 1.56–7.30; $P = 0.002$ for high vs. low cytokine class). Adjusting for living on a farm did not change the magnitude of the effect (OR, 3.56; 95% CI, 1.34–9.48; $P = 0.011$).

Gut Microbiome, Cytokine Classes, and Wheeze

Multivariate stepwise analyses differentiated between exposure to farm milk and exposure to barns or stables (i.e., between oral and inhaled exposures) with only consumption of farm milk remaining in the final model. This led us to hypothesize that the gut microbiome at 1 year might influence both cytokine classes and the association with wheeze in the first year of life in children at risk of asthma and might thus act as a mediator of the farm milk effect. Therefore, we assessed various dimensions of the compositional structure of the early gut microbiome and assessed their association with cytokine classes.

Of the first three axes from PCoA of the gut microbiome at 1 year, only the first PCoA axis—which correlated positively with the relative abundance of *Rikenellaceae*, *Ruminococcaceae*, *Faecalibacterium*, and *Roseburia* (Figure E2)—was significantly associated with the high cytokine class in 17q21 risk allele carriers (Table 3). No effects were seen in the total population (Table E5). Furthermore, the microbial diversity (i.e., Shannon index and species richness) showed a significant elevated effect on the high cytokine class. When defining "high-risk" not as 1 or more 17q21 risk alleles but as homozygous risk allele carriers, the microbial effects became stronger, with the effect of the butyrate score reaching statistical significance for the high cytokine class (Table E6). In contrast, the butyrate gene assay showed no effect, irrespective of genotype, potentially owing to small numbers ($n = 77$, data not shown). Interestingly, when testing for associations of single taxa with cytokine class in risk allele carriers, the same taxa that played a role in butyrate production and bacterial maturation (i.e., *Coprococcus* and *Roseburia*) were relevant (data not shown) (23).

Piecing the Puzzle Together: Mediation Analyses

To further disentangle the associations of farm milk consumption, fecal microbiome, and cytokine class, we conducted a mediation analysis in risk allele carriers to assess whether the effect of farm milk consumption on cytokine class was mediated by the fecal microbiome (Figure 3). Indeed, the indirect effect of farm milk depicted by the path from exposure through fecal microbiome to high cytokine class was significant ($P = 0.042$), indicating that the effect of farm milk on cytokine class is

Table 2. Factors Associated with Cytokine Classes in 17q21 Risk Allele Carriers

Exposure Variable	*n*	Cytokine Class	OR	95% CI	*P* Value
Characteristics at birth					
Farm child	126/257	Intermediate vs. low	0.91	0.49–1.69	0.774
		High vs. low	1.53	0.77–3.03	0.223
Maternal farm exposures in pregnancy					
Regular stay in stables	130/246	Intermediate vs. low	1.06	0.57–1.97	0.855
		High vs. low	2.70	1.28–5.67	**0.009**
Regular stay in barn	96/245	Intermediate vs. low	1.13	0.59–2.18	0.714
		High vs. low	2.96	1.43–6.15	**0.004**
Farm animal species, *n*					
0 (ref.)	104/255	Intermediate vs. low	1.00	—	—
		High vs. low	1.00	—	—
1	87/255	Intermediate vs. low	1.36	0.67–2.76	0.391
		High vs. low	1.10	0.47–2.57	0.825
⩾2	64/255	Intermediate vs. low	1.66	0.72–3.83	0.235
		High vs. low	2.37	1.01–5.54	**0.046**
Any farm milk consumption	111/257	Intermediate vs. low	1.28	0.69–2.36	0.440
		High vs. low	2.22	1.12–4.41	**0.022**
Farm exposures at 1 year of life					
Regular stay in stables	50/248	Intermediate vs. low	1.24	0.55–2.81	0.608
		High vs. low	2.26	1.00–5.14	**0.050**
Regular stay in barn	50/248	Intermediate vs. low	0.89	0.37–2.15	0.798
		High vs. low	2.04	0.86–4.82	0.105
Contact with hay	22/251	Intermediate vs. low	0.73	0.18–2.86	0.647
		High vs. low	2.94	1.05–8.26	**0.041**
Any farm milk consumption	76/248	Intermediate vs. low	1.18	0.59–2.39	0.637
		High vs. low	3.06	1.45–6.44	**0.003**
Analysis in milk drinkers only					
Milk consumption					
Any UHT milk (ref.)	31/165	Intermediate vs. low	1.00	—	—
		High vs. low	1.00	—	—
Any pasteurized milk	58/165	Intermediate vs. low	1.33	0.43–4.13	0.624
		High vs. low	1.71	0.38–7.72	0.485
Any farm milk	76/165	Intermediate vs. low	1.36	0.48–3.87	0.566
		High vs. low	3.75	1.06–13.25	**0.040**
Other exposures at 1 year of life					
Food diversity score					
0–8 items (ref.)	51/248	Intermediate vs. low	1.00	—	—
		High vs. low	1.00	—	—
9–11 items	91/248	Intermediate vs. low	1.13	0.49–2.58	0.781
		High vs. low	1.32	0.47–3.67	0.596
12–15 items	106/248	Intermediate vs. low	1.24	0.54–2.85	0.612
		High vs. low	2.54	0.95–6.77	0.063

Definition of abbreviations: CI = confidence interval; OR = odds ratio; ref. = reference; UHT = ultrahigh temperature.
Results from multinomial logistic regression models with cytokine class as three-categorical outcome and low cytokine class as reference category; results are adjusted for center. Statistically significant *P* values are given in bold.

partially mediated by the fecal microbiome with 23% of the total effect of farm milk being mediated by the first PCoA axis of the fecal microbiome.

Discussion

Data from the rural PASTURE birth cohort suggest a complex interaction of genetics, diet, the gut microbiome, early immune responses after innate stimulation, and disease, indicating that a multitude of dimensions matter for wheeze and asthma development. In this exploratory analysis of our high-dimensional data, the 17q21 risk allele was no longer associated with an increased risk of early wheezing or asthma at school-age among children with an early-activated immune response after innate LPS stimulation. The activation of the early immune response was associated with an oral but not inhaled farm exposure (i.e., consumption of unprocessed cow's milk [farm milk]). This beneficial effect was in turn partially mediated by the gut microbiome.

Children at Risk of Asthma

We defined children at risk of asthma based on the 17q21 gene locus, which has repeatedly been related to childhood asthma, particularly early in life (6–10). A cluster of SNPs in this region is associated with childhood-onset asthma, and the SNP used here is a good representative of this cluster, as recent data suggest that *GSDMB* may play a major role (8, 9, 14, 24). Thus, as a marker for elevated asthma risk in children in the current analyses, we used SNP rs7216389 in the *GSDMB* gene, which was in strong linkage disequilibrium with other SNPs in this region. Indeed, in our population, children with at least one risk allele of the rs7216389 SNP were at almost four times the risk of being asthmatic at age 6 years as

Table 3. Effect of Fecal Microbiome at Age 1 Year on Cytokine Classes in 17q21 Risk Allele Carriers ($n = 209$)

Fecal Microbiome (*z*-Scores)	Cytokine Class	OR	95% CI	*P* Value
Principal coordinate analysis				
First axis	Intermediate vs. low	1.04	0.72–1.50	0.834
	High vs. low	1.90	1.22–2.95	**0.004**
Second axis	Intermediate vs. low	0.86	0.59–1.26	0.443
	High vs. low	0.85	0.55–1.30	0.455
Third axis	Intermediate vs. low	1.11	0.76–1.63	0.583
	High vs. low	1.00	0.65–1.54	0.990
Shannon diversity index	Intermediate vs. low	1.11	0.76–1.63	0.594
	High vs. low	1.58	1.03–2.43	**0.035**
Species richness	Intermediate vs. low	1.10	0.72–1.67	0.657
	High vs. low	1.63	1.04–2.54	**0.033**
Estimated microbiome age	Intermediate vs. low	1.06	0.74–1.52	0.734
	High vs. low	1.47	0.92–2.33	0.103
Butyrate score	Intermediate vs. low	0.72	0.50–1.05	0.088
	High vs. low	1.33	0.95–1.86	0.094

Definition of abbreviations: CI = confidence interval; OR = odds ratio.
Results from multinomial logistic regression models with cytokine class as three-categorical outcome and low cytokine class as reference category; results are adjusted for center. Statistically significant *P* values are given in bold.

compared with children with no risk allele. Not surprisingly, the association with wheeze in the first year of life was much weaker, as most of the early wheezers in the PASTURE cohort were classified as transient or intermediate phenotypes, with no or almost no symptoms at age 6 years (22).

Deficient Responsiveness to Innate Stimuli

In the PASTURE birth cohort, we investigated the early immune response based on cytokine measurements after stimulation with the potent innate microbial trigger, LPS. Only those at-risk children without activation of Th1/Th2/Th17 cytokines after LPS were significantly at risk of early wheeze or asthma by the age of 6 years. In other words, a strengthened innate-induced activation in the first year of life protected from asthma development in carriers of the 17q21 risk alleles. The cytokines represented in the three latent classes are related to both innate and adaptive immunity. In fact, all "innate" cytokines (IL-1β, IL-6, and TNF-α) were detectable in almost all children after LPS stimulation and were thus excluded from latent class analysis. The intermediate class was characterized by cytokines related to Th1 (IL-12p70 and IFN-γ) and to a lesser extent to Th17 (IL-17) responses. Interestingly in this class, although leveling out the risk of wheeze to some extent in the at-risk population, the effect was not as pronounced as in the highest cytokine class, suggesting that the Th1/Th2 paradigm does not explain our results. The highest class in turn included cytokines related to Th1, Th2, and Th17 immune responses. A sensitivity analysis including the highly detectable innate cytokines in analysis resulted in almost identical allocation of the children to the three latent classes. Furthermore, when using higher than detection level cutoffs for either the highly-detectable cytokines or all cytokines (e.g., lowest decile, quartile, or median), the resulting latent classes showed no comparable interaction with asthma risk and were not associated with any environmental exposures (data not shown). These findings indicate that responsiveness to the potent innate stimulus LPS at the interface to adaptive immunity, rather than concentrations of individual innate or Th1/Th2/Th17–associated cytokines matter. Low Th1/Th2/TH17 responses after innate stimulation may reflect an inefficient innate-adaptive crosstalk in the first year of life, which in turn confers risk of school-age asthma in 17q21 risk allele carriers. In contrast, more robust immune responses to LPS may be relevant for two reasons: first, a propensity of early life immune activation via TLR signaling can counterbalance potentially harmful immune responses genetically set through a risk genotype. Evidence for this hypothesis of robust immune responses to LPS is provided by some recent publications: We have shown that *ex vivo* farm dust or LPS stimulation can restore TNFAIP3 expression, an antiinflammatory protein within the NF-κB signaling pathway, to healthy amounts. This was feasible even in people with manifest asthma and shifted NF-κB signaling–associated gene expression toward an antiinflammatory state (25). Second, LPS or farm dust stimulated not only NF-κB signaling, but also the MAPK signaling pathway. In this context, farm-dust stimulation was associated with increased DUSP1 expression, a negative regulator of the MAPK signaling pathway for inflammation, reaching healthy levels and downregulated inflammatory MAPKs (26). Both reports show that potent LPS- (or farm dust–) induced immune response can effectively counteract proinflammatory responses. Whether our observations are generalizable from LPS to other innate stimuli must, however, be further investigated.

Innate Immunity in Farming Environments

Innate stimulation from environments rich in microbial exposures has been shown in a number of previous cross-sectional farm studies. For example, increased gene expression of TLR downstream signaling molecules such as IRAK-1, IRAK-2, and RIPK1, as well as HLA-DRA and SOCS-4, was found among farm children, whereby the expression of IRAK-1, IRAK-2, and RIPK1 partially mediated the protective farm effect on asthma (27). These data suggest that activation of innate immunity is associated with both farm exposure and reduced asthma risk. In the Amish farm population, a strong reduction in the prevalence of asthma and atopy was found. Furthermore, the

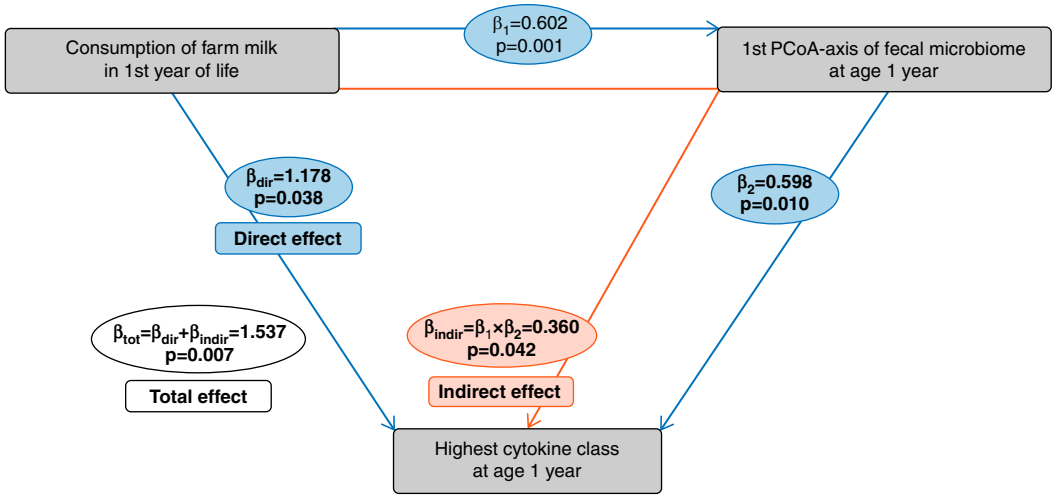

Figure 3. Mediation of the effect of farm milk consumption on cytokine class by the fecal microbiome in children at risk of asthma (17q21 risk allele, $n = 201$). The total effect of farm milk consumption on high cytokine class (versus intermediate or low class) at age 1 year in children at risk of asthma is divided into a direct and an indirect path (estimates and *P* values of each effect are indicated separately). The first axis from principal coordinate analysis of the fecal microbiome at age 1 year is shown as a possible indirect link between farm milk exposure and high cytokine class. All effects are adjusted for farming and center. Direct paths are shown in blue; indirect paths are shown in red. Statistically significant estimates and *P* values are given in bold. dir = direct; indir = indirect; PCoA = principal coordinate analysis; tot = total.

protective effect of Amish environmental exposure on experimental allergic airway disease disappeared in MYD88/TRIF knockout mice, suggesting that childhood asthma is associated with deficiencies in the innate immune response and that specific environments may provide protection against asthma by innate immune activation (5). Our findings refine this concept, suggesting that the interaction between innate and adaptive responses after microbial (LPS) stimulation may particularly matter for asthma development in 17q21 risk allele carriers.

It is noteworthy that in our analyses, even though almost all farm-related exposures were significantly associated with an innate-induced activated immune response in univariate analyses, only the consumption of unprocessed cow's milk remained in the final farm-adjusted multivariate model. This potentially indicates that not inhaled exposures, such as stable contact, are essential for activation of circulating immune cells after LPS, but rather dietary features and associated characteristics of the early gut microbiome. These results support the notion of a gut–immune axis in very young children. In contrast, the previously reported protective effect of inhaled exposure to stables in 17q21 risk allele carriers on wheeze is presumably transmitted by mucosal mechanisms rather than by a systemic immune response (14). These results underline the multitude of mechanisms by which farm exposures early in life may protect from asthma: both stable exposure and farm milk consumption are independent protective factors for the development of disease. However, only 23% of the milk effect was mediated by the gut microbiome, indicating additional protective mechanisms. Potentially epigenetic mechanism like DNA methylation, histone modification, or microRNAs contribute to the effects induced by unprocessed milk (28). It remains, however, unclear which ingredients in farm milk exert the observed beneficial effect. The observation that boiling did not alter the effect suggests that heat stabile compounds such as fatty acids, in particular ω-3 polyunsaturated fatty acids, which are precursors of antiinflammatory mediators (29), and oligosaccharides acting as prebiotics may alter the compositional structure of the gut microbiome and thereby boost the early immune response. Other milk components like bovine IgG or TGF-β might also play a role: the former can bind to bacterial and viral pathogens and enhance phagocytosis and may neutralize pathogens, whereas the latter promotes epithelial barrier functioning and might favor the differentiation of Tregs that can reduce inflammation locally (30). A recent review on the impact of raw milk on the immune system in early life summarized that the industrial processing of milk and dairy products, needed to ensure microbiological safety, typically results in denatured milk proteins, which lose their functional activity, suggesting that preserving milk proteins and preventing glycation may be important innovations to help prevent future disease (28).

The Immunomodulatory Role of the Gut Microbiome

Alterations in the compositional structure of the gut microbiome such as decreased diversity, inadequate maturation, and lower abundance of taxa producing short-chain fatty acids have all been incriminated as conferring asthma risk (31–33), and bacterial metabolites have often been found to be associated with health effects (34). Data from animal models further point to the importance of bacterial metabolites transmitting signals from the gut to the bone marrow, thereby shaping immune responses (35). These experimental studies also demonstrate that early life represents a critical window during which the gut microbiome can shape systemic immune function later in life (36).

We have recently shown for the PASTURE cohort that accelerated maturation of the gut microbiome in the first year of life was directly associated with decreased asthma risk at 6 years (23). Here we report that the compositional structure of the gut microbiome by the age of 1 year, but not the microbiome maturation, indirectly impacts asthma development in risk allele carriers via activated immune responses after innate stimulation. Thus, different facets of the gut microbiome may have an impact on

the gut–lung and the gut–immune axis, respectively, all eventually directly and indirectly contributing to decreased asthma risk. Common to both pathways may be the microbial capacity for production of short-chain fatty acids such as butyrate.

Strengths and Limitations

The major strength of the PASTURE study is its unique nature as a birth cohort within a rural farming environment, an environment that has already changed since the beginning of the study in 2002. Furthermore, the multitude of measurements assessed in early life, such as genetics, cytokines, and fecal microbiome enable a quite comprehensive analysis of the early origins of childhood asthma. Nevertheless, the number of children with all measurements available at age 1 year and with a follow-up at age 6 years was fairly small, leading to small numbers for several analyses, even more so as the asthma prevalence in our rural population was comparatively low. Moreover, our analyses are of exploratory character and would benefit from a confirmation in a replication cohort.

Conclusions

Our findings from the rural PASTURE birth cohort indicate that asthma development in 17q21 risk allele carriers is associated with an impaired immune responsiveness to the potent innate stimulus LPS. In contrast, risk allele carriers with an activated Th1/Th2/Th17 immune response after innate stimulation by 1 year were at no increased risk of wheeze and asthma up to age 6 years. Oral rather than inhaled exposures and the resulting changes in the compositional structure of the early gut microbiome restored responsiveness to microbial LPS. Based on these results from our exploratory analyses, we speculate that within the 17q21 genotype, asthma risk might be mitigated by restoring Th1/Th2/Th17 activation after microbial stimulation. ■

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PASTURE Study Group members: Andreas Böck (Dr. von Hauner Children's Hospital, Ludwig Maximilians University Munich, Munich, Germany); Markus J. Ege (Dr. von Hauner Children's Hospital, Ludwig Maximilians University Munich, Munich, Germany; Institute of Asthma and Allergy Prevention, Helmholtz Zentrum München, Neuherberg, Germany; member of the German Center for Lung Research, Germany); Remo Frei (Christine Kühne Center for Allergy Research and Education, Davos, Switzerland; Division of Respiratory Medicine, Department of Paediatrics, Inselspital, University of Bern, Bern, Switzerland); Jon Genuneit (Ulm University, Institute of Epidemiology and Medical Biometry, Ulm, Germany; Pediatric Epidemiology, Department of Pediatrics, University Medicine Leipzig, Leipzig, Germany); Lucie Laurent (University of Besançon, Department of Respiratory Disease, UMR/CNRS6249 Chrono-environment, University Hospital, Besançon, France); Sonali Pechlivanis (Institute of Asthma and Allergy Prevention, Helmholtz Zentrum München, Neuherberg, Germany); Martin Täubel (Department of Health Security, Finnish Institute for Health and Welfare, Kuopio, Finland); and Johanna Theodorou (Dr. von Hauner Children's Hospital, Ludwig Maximilians University Munich, Munich, Germany; member of the German Center for Lung Research, Germany).

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