

Association of *NOD1* polymorphisms with atopic eczema and related phenotypes

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Background: Interactions with microbial pathogens are crucial for the maturation of the immune system. The nucleotide-binding oligomerization domain protein 1 (*NOD1*) is a cytosolic receptor sensing a muropeptide found mostly in gram-negative bacterial peptidoglycans. *NOD1* is located on chromosome 7p14-p15, a region that has been linked with atopy. Recently, polymorphisms of the closely related *NOD2* have been associated with atopy-related traits.

Objectives: Within a large population-based cohort of German adults ($n = 1417$), a case-control population for atopic eczema ($n = 454$), and a large cohort of parent-offspring trios for atopic eczema (189 trios), we evaluated 11 *NOD1* polymorphisms for associations with atopic phenotypes.

Methods: Subjects were phenotyped by standardized questionnaires and interviews, skin examination, and serum IgE measurements. Genotyping was performed by using matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Results: Analyses revealed significant association of one *NOD1* haplotype with atopic eczema in the population-based cohort ($P = .004$) and the case-control population ($P = .003$). Another

NOD1 haplotype was associated with decreased total IgE ($P = .008$). In addition, significant associations with total serum IgE levels were observed for polymorphisms rs2907748 ($P = .006$), rs2907749 ($P = .012$), and rs2075822 ($P = .018$). These polymorphisms were significantly associated with atopic eczema and asthma in the family-based association analyses ($P = .001-.043$). Seven polymorphisms showed significant transmission distortion for total IgE levels (P values $< .0001-.029$).

Conclusion: These data indicate that genetic variants within *NOD1* are important determinants of atopy susceptibility. (J Allergy Clin Immunol 2005;116:177-84.)

Key words: Atopic eczema, asthma, allergic rhinoconjunctivitis, IgE, *CARD4*, *NOD1*, SNP, haplotype, association

Atopic diseases (asthma, rhinoconjunctivitis, atopic eczema) are complex polygenic disorders influenced by gene-environment interactions. Over the last decades, their prevalence has shown a steady increase in Western countries, whereas there is a considerably lower prevalence in developing countries.¹ Several epidemiological studies (see review²) suggest that reduced microbial exposure in early childhood as a result of Western lifestyle factors is mainly responsible by causing a bias toward T_H2 responses, a theory known as the hygiene hypothesis.³ However, the prevalence of T_H1 -dominated immune disorders like insulin-dependent diabetes mellitus, multiple sclerosis, and inflammatory bowel disease has also increased in developed countries,⁴ and T_H1 -driven and T_H2 -driven diseases are not mutually exclusive.⁵⁻⁸

Therefore, the hygiene hypothesis has been modified, and inappropriate counterregulation by T-regulatory cells, which might permit patterns of immune response predisposing to either autoimmunity or allergy depending on the individual genetic susceptibility, has been emphasized.⁹

Innate immunity represents the first line of host defense recognizing a few highly conserved structures present in many different microorganisms. These pathogen-associated molecular patterns (PAMPs) are recognized through a set of germline-encoded pattern recognition receptors (PRRs). PRRs located in the cell membrane such as Toll-like receptors (TLRs) or CD14 respond to extracellular PAMPs. Cytosolic PRRs like *NOD1* (also designated *CARD4*) and *NOD2* (also designated *CARD15*) recognize PAMPs that cross the plasma membrane (see review^{10,11}).

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Abbreviations used

CARD: Caspase recruitment domain-containing protein
 KORA: Cooperative Health Research in the Region of Augsburg
 NOD: Nucleotide-binding oligomerization domain protein
 OR: Odds ratio
 PAMP: Pathogen-associated molecular pattern
 PRR: Pattern recognition receptor
 SNP: Single nucleotide polymorphism
 TLR: Toll-like receptor
 TDT: Transmission/disequilibrium test

NOD1 belongs to a family of nucleotide-binding oligomerization domain proteins and is composed of an N-terminal caspase recruitment domain, a centrally located NOD, and multiple C-terminal leucine-rich repeats, and is expressed constitutively by epithelial cells.^{10,11} The CARD domain and the nucleotide-binding domain have been shown to cause nuclear factor- κ B activation via the serine/threonine kinase RICK (RIP-like interacting CLARP kinase, also called *Rip2* and *CARDIAK*),¹² whereas the leucine-rich repeats interact with specific bacterial peptidoglycan muropeptides.¹³

Genetic variations in innate immunity genes have been reported to be associated with a range of inflammatory disorders, including both T_H2 -driven atopic and T_H1 -dominated autoimmune diseases (see review¹⁴).

Polymorphisms in the *NOD2* gene have been identified at the inflammatory bowel disease 1 locus. These DNA variants have been found to be associated with Crohn disease and involved in the etiology of the disease.¹⁵⁻¹⁷ Recently, it was shown that *NOD2* single nucleotide polymorphisms (SNPs) are also associated with atopic phenotypes.¹⁸

Because *NOD1* shares many structural and functional similarities with *NOD2* and is located on chromosome 7p14-p15, a region which was reported to contain an atopy susceptibility locus,¹⁹ we investigated 11 *NOD1* polymorphisms covering the whole gene and exhibiting allele frequencies reasonable for analysis for associations with atopy-related phenotypes using 3 independent cohorts: a large population-based cross-sectional cohort of adults with a focus on allergic sensitizations and respiratory atopy, a case-control population for atopic eczema, and a cohort of parent-offspring trios for atopic eczema.

METHODS

Study population

Three independent cohorts were examined in this study: a population-based cohort (Cooperative Health Research in the Region of Augsburg [KORA] C, 1417 individuals), a case-control cohort for atopic eczema (KORA S4, 454 individuals), and a trio cohort for atopic eczema (189 trios).

The KORA C and KORA S4 surveys are large population-based cross-sectional studies on adults performed from 1994 to 1995 and from 1999 to 2001 in the city and region of Augsburg, South Germany. The sampling frames and study designs have been described previously.^{20,21}

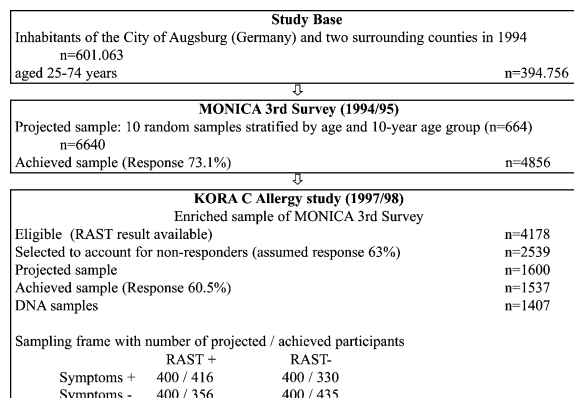


FIG 1. Design and sampling frame of the KORA C study.

Briefly, KORA C is based on a random, cross-sectional sample stratified for age and sex studied in 1994/1995 (n = 4356). In the sera of these subjects, allergen-specific IgE antibodies to common aeroallergens (grass and birch pollen, house dust mite, cat, and *Cladosporium*) were determined by the fluorescence enzyme immunoassay technique (CAP-FEIA; Pharmacia, Uppsala, Sweden). The cutoff point for RAST reactivity was set at 0.35 kU/L. For KORA C, we aimed to recruit an enriched sample of 1600 subjects out of the 4178 individuals who had valid RAST results.

Subjects were selected, stratified by age and sex, to provide 50% with and 50% without a positive RAST, and so that within these groups, 50% reported respiratory atopy (n = 1417). To account for an estimated response of 63%, a sample of 2539 subjects was approached. Finally, 1537 subjects participated (60.5%), of whom 50.2% exhibited a positive RAST result, and 53.9% of this group and 43.1% of those with a negative RAST result reported symptoms of atopy. DNA samples were available from 1407 subjects. Details of the sampling frame of KORA C are given in Fig 1.

KORA S4 studied an independent population-based cohort of 4261 subjects. From KORA S4, 227 individuals with atopic eczema and 227 matched controls were selected.

For family-based association analyses, we used 189 complete white parent-offspring trios for atopic eczema who had been recruited between January 2001 and December 2003 in Munich and Bonn, Germany.

All study methods were approved by the ethics committee of the Bayerische Landesärztekammer Munich.

A written and informed consent was obtained from all participants before the beginning of the study.

Phenotyping

All subjects had to complete a standardized questionnaire that, in addition to demographic data, included the basic allergy questions of the European Community Respiratory Health Survey on respiratory health.²² In addition, all probands were interviewed in a standardized manner to report on symptoms of atopic diseases, basic family data, and parental history of atopic diseases.

In all subjects, total as well as specific IgE antibodies against common environmental allergens (grass, birch, rye, mugwort pollen, *Alternaria*, *Cladosporium*, cat, dog, and *Dermatophagoides pteronyssinus*) were measured (enzyme immunoassay: CAP-FEIA). Subjects were classified as having asthma, allergic rhinoconjunctivitis, or atopic eczema when they reported a physician's diagnosis of asthma, rhinoconjunctivitis, or atopic eczema, respectively. Specific sensitization was defined to be present if at least 1 of the specific IgE antibodies was positive (CAP class ≥ 1 , corresponding to ≥ 0.35 kU/L). Elevated IgE was defined as a qualitative trait with serum

concentration above the 50th percentile. Affected offspring of the trio study were classified as having atopic eczema on the basis of a skin examination by experienced senior dermatologists using the UK diagnostic criteria for atopic eczema.²³

Genetic analyses

Single nucleotide polymorphism information for this article was retrieved from the Innate Immunity Programs for Genomic Applications, National Heart, Lung, and Blood Institute Program in Genomic Applications (innateimmunity.net/data/homology).

Genotyping analyses were performed by using the MassARRAY system (Sequenom, San Diego, Calif). Briefly, genomic DNAs were amplified by PCR using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Genotyping assays were performed by using 5 ng genomic DNA. PCR primers were used at 167 nmol/L final concentrations for a PCR volume of 6 μ L. The PCR condition was 95°C for 15 minutes for hot start, followed by denaturing at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute for 45 cycles, and finally incubation at 72°C for 10 minutes. PCR products first were treated with shrimp alkaline phosphatase (Amersham, Freiburg, Germany) for 20 minutes at 37°C to remove excess deoxyribonucleoside triphosphates and afterward for 10 minutes at 85°C to inactivate shrimp alkaline phosphatase. Thermo-Sequenase (Amersham) was used for the base extension reactions. Extension primers were used at a final concentration of 5.4 μ mol/L in 10- μ L reactions. The base extension reaction condition was 94°C for 2 minutes, followed by 94°C for 5 seconds, 52°C for 5 seconds, and 72°C for 5 seconds for 40 cycles. All reactions (PCR amplification, base extension) were performed in a Tetrad PCR thermal cycler (MJ Research, Waltham, Mass). The final base extension products were treated with SpectroCLEAN resin (Sequenom) to remove salts in the reaction buffer. This step was performed with a Multimek 96-channel autopipette (Beckman Coulter, Fullerton, Calif.), and 16 μ L resin-water suspension was added into each base extension reaction, making the total volume 26 μ L. After a quick centrifugation (2000 rpm, 3 minutes) in an Eppendorf Centrifuge 5810, 10 nL reaction solution was dispensed onto a 384 format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroPoint nanodispenser (Sequenom). A modified Bruker Biflex matrix-assisted laser desorption ionization time of flight mass spectrometer (Sequenom) was used for data acquisitions from the SpectroCHIP. Genotyping calls were made in real time with MASSARRAY RT software (Sequenom). For analysis of sequence binding sites, MatInspector release 7.0 (Genomatix, Munich, Germany) was used.

Statistics

Descriptive statistics for quantitative and qualitative values are given by means $\pm$ SDs and relative frequencies or absolute numbers, respectively. For descriptive statistics, SPSS version 11.5 (SPSS Inc, Chicago, Ill) was used. Association of quantitative traits with SNP genotype was tested with *t* tests for 2 independent samples or Satterthwaite tests by using the SAS statistical software package version 9.1 (SAS Institute, Cary, NC). Deviation from Hardy-Weinberg equilibrium was tested with PROC ALLELE in SAS/Genetics for any of the SNPs under consideration. Linkage disequilibrium was assessed with procedure ldmax in the software package GOLD (availability: <http://www.well.ox.ac.uk/asthma/GOLD>). Haplotype frequencies were estimated from genotype data by using the Expectation Maximization (EM) algorithm.^{24,25} To evaluate associations with quantitative and qualitative traits, haplotype trend regression models were applied, including estimated probabilities of haplotypes in a linear and logistic regression approach as independent variables,²⁵ respectively. Further independent variables were study

population (KORA C or KORA S4), age, family size, and sex. Because smoking is an essential influence factor on IgE levels and atopic diseases,²⁶ smoking status was included as independent variable in multiple regressions.

The same covariates were included in logistic regressions testing associations of qualitative traits with SNP genotypes. Results are reported after applying backward elimination and stepwise variable selection. All haplotype analyses were implemented by using the SAS/Genetics module. Odds ratios (ORs) are given with 95% CIs and 2-sided *P* values in parentheses.

Family-based association analyses were performed using 189 trios. The PedCheck program (O'Connell JR and Weeks DE, 1998) was used to exclude Mendelian inconsistencies in allele assignment in each nuclear family.²⁷ Evidence of association with the binary traits atopic eczema, asthma and rhinoconjunctivitis was evaluated using the classic transmission/disequilibrium test (TDT)²⁸ implemented in SAS/Genetics. Transmitted and untransmitted allele frequencies as well as contingency tables for TDT analyses were calculated using SAS version 9.1. *P* values for TDT analyses were obtained from the conditional exact McNemar test using STATXact version 5 (Cytel Software, Cambridge, Mass). Quantitative, log-transformed total IgE values were assessed with the quantitative version of the TDT as previously described and implemented.²⁹ Any *P* values given are 2-sided and not adjusted for multiple comparisons, because the comparisons do not represent completely independent tests, and replication of results was achieved in 3 independent cohorts. However, association results in the family-based analyses remained significant after applying Bonferroni correction for multiple testing.

The patterns of linkage disequilibrium between markers were visualized using the GOLD 1.0 package.

RESULTS

The location of the genotyped *NOD1* SNPs and their frequencies are provided in Table I and Fig 2. All analyzed SNPs were in Hardy-Weinberg equilibrium, and analysis of Mendelian inheritance within the families revealed no major errors. Calculation of the linkage disequilibrium in the gene showed a moderate to strong degree of LD (*D'*) across the gene (Fig 3).

Phenotypic characteristics of all investigated individuals are summarized in Table II.

In KORA C, between 1306 and 1393 samples (average call rate, 96.4%) were genotyped successfully for the respective *NOD1* polymorphisms.

The presence of a C allele at rs2907748, an A allele at rs2907749, and a T allele at position rs2075822 revealed to be significantly associated with elevated IgE levels, with ORs of 1.36 (95% CI, 1.09-1.7; *P* = .006), 1.30 (95% CI, 1.06-1.59; *P* = .012), and 1.32 (95% CI, 1.05-1.66; *P* = .018), respectively. For homozygosity at polymorphisms rs2907748 (CC), rs2907749 (AA), and rs2075822 (TT), we observed ORs of 3.07 (95% CI, 1.61-5.85; *P* = .0006), 1.63 (95% CI, 1.01-2.64; *P* = .046), and 3.23 (95% CI, 1.56-6.68; *P* = .0016). In KORA C, genotypes AA at rs2736726 and GG at rs2075817 were weakly associated as protective for atopic eczema, with ORs of 0.41 (95% CI, 0.18-0.94; *P* = .036) and 0.43 (95% CI, 0.18-0.99; *P* = .046). No significant associations with specific sensitization and allergic rhinoconjunctivitis were seen in the single locus analyses (data not shown).

TABLE I. *NOD1* polymorphisms, database SNP accession numbers, localization, genotype frequencies, and genotyping primers

SNP ID	Location	Genotype	Frequency	Direction	PCR primer	Extension primer
rs2736726	5' flanking	AA	0.075	Forward	ACGTTGGATGGTCCATAAGGGATATCATGC	GAATGTACTTCTTC
		AG	0.357	Reverse	ACGTTGGATGTACTCACAAGCATCAGTGGC	AGTGTGA
		GG	0.568			
rs2075817	Intron 1	AA	0.553	Forward	ACGTTGGATGAGGCCCTTTCTGAGGTCCTG	CGTTCCGCCCGCAC
		AG	0.368	Reverse	ACGTTGGATGTTTCTCCGCTTGGTTCCACC	GCA
		GG	0.079			
rs2975632	Intron 2	CC	0.047	Forward	ACGTTGGATGCTTTAGAAAGCTGGGAAGGG	GAGGGATGAAGGA
		CT	0.319	Reverse	ACGTTGGATGAAGCAGTGAGTAAACGGGC	AGCTG
		TT	0.634			
rs3020207	Intron 3	AA	0.545	Forward	ACGTTGGATGTAGAGAAACCCACAACCAG	GCAAAAGCTACAG
		AG	0.380	Reverse	ACGTTGGATGCCCCTGAAGAGATCAATGAC	AAAGAGC
		GG	0.074			
rs2075818	Exon 4	CC	0.571	Forward	ACGTTGGATGCAGGCACACACAATCTCCGC	GCTGAAGAATGACT
		CG	0.366	Reverse	ACGTTGGATGCCGCAATACTCAGTGTCTGG	ACTTCTCGGC
		GG	0.063			
rs2235099	Exon 6	CC	0.570	Forward	ACGTTGGATGTGCTATGCCCAAGAGGAGGAG	AACCAGCTCCATGA
		CT	0.371	Reverse	ACGTTGGATGCATTGCTGAAGCCAACCAGC	TGGT
		TT	0.064			
rs2075821	Exon 6	AA	0.075	Forward	ACGTTGGATGTATCCTCCCTTCCTCCCGTTC	CTTGAAGAGGTCTT
		AG	0.361	Reverse	ACGTTGGATGGGAAGAGGTTGGTGAAGTGG	CCCC
		GG	0.565			
rs2075822	Intron 6	CC	0.044	Forward	ACGTTGGATGAAGGGCCATGGTCATGAGTC	GGGCATCGGGAAT
		CT	0.335	Reverse	ACGTTGGATGACTGTTCTCAGGTGAGGCTG	GGCA
		TT	0.622			
rs2907749	Intron 9	AA	0.542	Forward	ACGTTGGATGAATCTCTGAGGTTGGGTGAG	CAGGTTGTACCACA
		AG	0.378	Reverse	ACGTTGGATGTGAAAAGAAGCAAGGCCC	TACATCC
		GG	0.080			
rs2907748	Intron 11	CC	0.586	Forward	ACGTTGGATGAATCTGAGGGCTCAGCAAAG	CCTGGGCTCCTATT
		CT	0.362	Reverse	ACGTTGGATGACAGAAGGTCAATGGGACTC	TCTTAGCC
		TT	0.052			
rs5743368	Intron 13	AA	0.026	Forward	ACGTTGGATGACCATGCACATTGCATCCTG	CTGATCGATTTTAT
		AG	0.267	Reverse	ACGTTGGATGAACACAGGCTCCTCATACAG	GACAAGTTC
		GG	0.711			

For haplotype analysis, 1191 samples were available in which genotyping was successful in each of the 11 *NOD1* polymorphisms. The EM algorithm showed 6 possible haplotypes exceeding a frequency of 3%. Ninety-five percent CIs for frequencies are small because of the large study population, indicating sufficiently high accuracy.

Analyses revealed that haplotype G-A-C-A-C-C-G-C-G-T-G is significantly protective against the development of elevated IgE levels (OR, 0.514; 95% CI, 0.315-0.840; $P = .008$).

We further observed that haplotype A-G-T-A-C-C-G-T-A-C-G demonstrated weak evidence for association with atopic eczema (OR, 0.260; 95% CI, 0.074-0.919; $P = .0037$). Because the number of patients with from atopic eczema in KORA C was low ($n = 28$), we aimed at validating this association in KORA S4, which was selected as a case-control population for atopic eczema ($n = 454$). Analyses in KORA S4 confirmed that this haplotype is significantly protective against the development of atopic eczema, with an OR of 0.119 (95% CI, 0.030-0.472; $P = .003$). This corresponds to results from

single analyses in which the presence of A (rs2736726) and G (rs2075817) alleles at respective positions was associated with decreased risk. Haplotype frequencies and association results are shown in Table III.

Family-based association analyses showed significant associations with the trait IgE modeled as quantitative trait in a regression model for 7 of the 11 SNPs ($P \leq .0001-.029$; Bonferroni corrected P value for all tests = .023), confirming the results of the large population-based study KORA C. Preferential transmission to atopic eczema-affected and asthma-affected children was seen for the rs2907748 C allele ($P = .001/.015$), rs2907749 A allele ($P = .032/.011$), and rs2075822 T allele ($P = .005/.024$) validating the association results found in KORA C and KORA S4, and with the rs2975632 T allele ($P = .043/.036$). No associations were seen for allergic rhinoconjunctivitis or specific sensitization (data not shown).

Because of haplotype ambiguity in a substantial proportion of families, the multilocus haplotypes could not be analyzed in the trio cohort.

Table IV provides an overview on the association results of the population-based and family-based analyses.

DISCUSSION

This study represents the first evaluation of the *NOD1* gene as an atopy susceptibility locus. Using a large population-based cross-sectional cohort of 1417 German adults, a case-control population for atopic eczema of 454 individuals, and an independently ascertained large cohort of 189 parent-offspring trios for atopic eczema, we analyzed the effects of 11 SNPs in the *NOD1* gene on atopic phenotypes. High-quality genotyping results were achieved for all of the SNPs with call rates >95%, Hardy-Weinberg equilibrium, and no major Mendelian inconsistencies.

Within the population-based analyses, significant associations of *NOD1* SNPs rs2907748, rs2907749, and rs2075822 with IgE levels could be observed. Polymorphisms rs2736726 and rs2075817 showed weak associations with atopic eczema. No associations were seen with allergic rhinoconjunctivitis and specific sensitization.

Because haplotype analyses may be of higher informative value to draw associations between phenotypes and genetic variation than SNPs,³⁰ we also assessed the effects of haplotypes in our population-based cohorts. Analyses revealed significant association between the *NOD1* haplotype G-A-C-A-C-C-G-C-G-T-G and IgE as well as haplotype A-G-T-A-C-C-G-T-A-C-G and atopic eczema.

The TDT and quantitative TDT also yielded significant results for these 2 traits. Seven out of 11 SNPs investigated were significantly associated with total serum IgE levels. Two of these significantly associated SNPs are located in the coding regions without causing an amino acid exchange. So far, their functional consequences are not clear.

Interestingly, in addition to rs2975632, SNPs rs2907748, rs2907749, and rs2075822, which were found to be associated with total serum IgE levels in the population-based analyses, showed significant transmission distortion for the related traits atopic eczema and asthma, but not for allergic rhinoconjunctivitis and specific sensitization.

These results indicate that the locus does not modulate the total serum IgE through an allergen-specific mechanism. It is consistent with observations that only a small portion of total serum IgE is directed against known allergens and that elevation of the total serum IgE may predict atopic diseases independently of the presence or absence of allergen-specific IgE, and it supports the observation that atopic traits, despite a considerable overlap, might also be influenced by unshared genetic determinants.³¹

Our study has several important aspects. We assessed 11 SNPs covering the complete *NOD1* gene (Fig 2), and associations could be detected in 3 large, independent populations that had been extensively phenotyped, suggesting that multiple testing or spurious associations on the basis of population stratification is not a major issue. Both individual SNPs and their common 11-locus haplotypes were evaluated. Finally, along with qualitative analyses of the dichotomous traits asthma, rhinoconjunc-

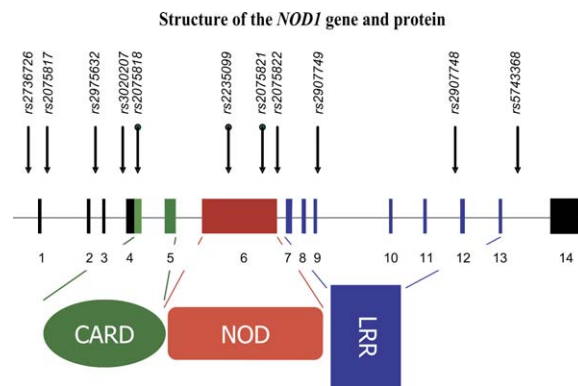


FIG 2. Structure of *NOD1* gene and protein. The *NOD1* gene extends more than 55 kb of genomic DNA and contains 14 exons. A 5' untranslated region (UTR) is located within exons 1-4, and a 3' UTR is found within exon 14. All polymorphisms analyzed are indicated by arrows (round-tailed arrows indicate exonic SNPs) and are described in public databases (www.innateimmunity.net). The *NOD1* protein consists of 965 amino acids and contains an N-terminal CARD, a centrally located NOD, and multiple C-terminal leucine-rich repeats (LRRs).

tivitis, and atopic eczema, the quantitative phenotype total serum IgE has been analyzed.

However, some limitations must be considered when interpreting our data. Although a large number of SNPs has been investigated, we did not perform complete sequencing and might therefore have missed other important variations. It cannot be ruled out that the observed associations are a result of the other SNPs not assessed by genotyping but in linkage disequilibrium. Some polymorphisms showed significant associations with different (yet closely related) traits in population-based analyses and TDT. Finally, because of haplotype ambiguity in a substantial number of trios, the multilocus haplotypes could not be analyzed in the trio cohort.

Atopic diseases arise through a combination of genetic and environmental factors, and T_H2 polarization and persistent accumulation of inflammatory cells are regarded as pathophysiologic characteristics (see review³²). As a possible cause for the increase in allergic diseases seen in Western countries over the past decades, an altered stimulation of innate immune responses during childhood leading to impaired regulatory T-cell development has attracted much attention.³³

Exposure to microbial stimuli is important for the postnatal development of the immune system, with innate immunity playing a crucial role in activating and directing adaptive immunity.¹¹ Epidemiologic data show that during early childhood, microbial exposure can influence the induction and expression of atopic diseases, particularly in the respiratory tract.³⁴ Increased endotoxin exposure has been associated with decreased sensitization rates^{35,36} and lower prevalences of respiratory atopy.³⁷ Whereas endotoxin occurs only in gram-negative bacteria, peptidoglycan is a major component of the cell wall of all bacteria. *NOD1* and *NOD2* respond to different motifs in peptidoglycan, and specificity for gram-negative

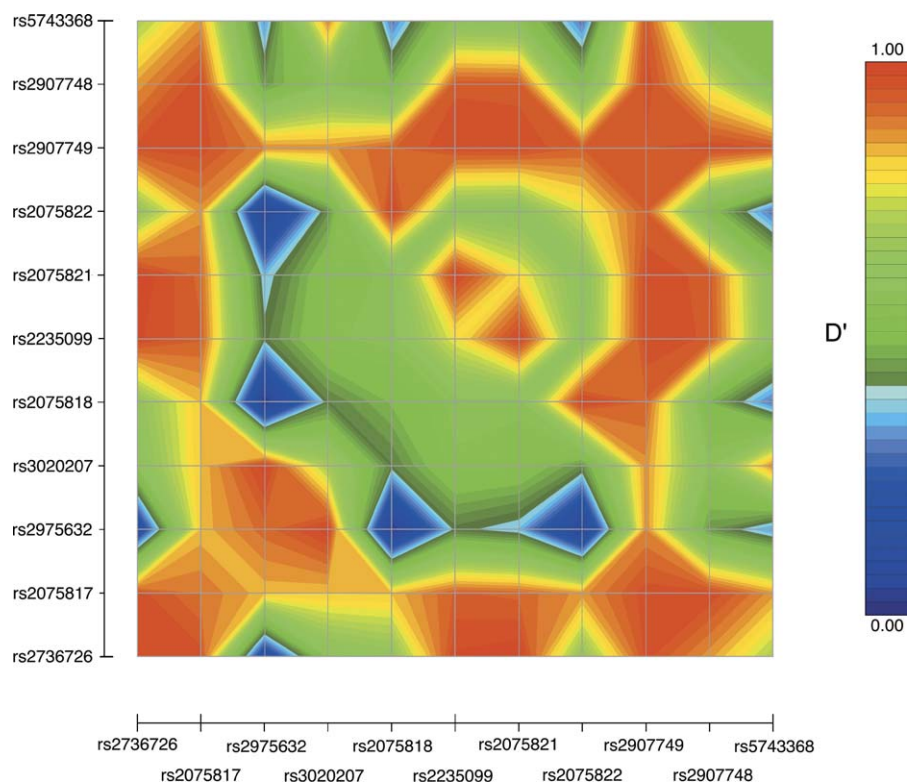


FIG 3. GOLD linkage disequilibrium map of the *NOD1* SNPs applying a normalized correlation coefficient. Pairwise estimations of D' are shown from unrelated subjects (KORA C) on a scale of 1 (complete linkage disequilibrium: red) to 0 (blue). Marker positions are shown as a schematic rather than as actual distances apart.

TABLE II. Phenotype characteristics of all individuals (KORA adults and trios) included in analyses

	KORA C (n = 1417)	KORA S4 (n = 454)	Trios (n = 189)	
			Parents	Offspring
Male sex, %	49.67	40.80	49.38	42.12
Mean ($\pm$ SD) age	50.48 ($\pm$ 13.28)	44.17 ($\pm$ 13.57)	44.2 ($\pm$ 9.2)	12.3 ($\pm$ 7.6)
Asthma, %	8.85	9.25	12.31	51.10
Atopic eczema, %	1.96	50.00	33.25	100
Allergic rhinoconjunctivitis, %	25.24	28.41	11.61	30.29
Mean ($\pm$ SD) ln (total serum IgE)	3.96 ($\pm$ 1.41)	3.85 ($\pm$ 1.41)	3.71 ($\pm$ 5.64)	5.56 ($\pm$ 5.28)

TABLE III. *NOD1* haplotypes, haplotype frequencies with 95% CIs (haplotypes with frequencies <3% were not considered), and association results†

Haplotype	KORA C				KORA S4			
	Frequency	95% CI	Association		Frequency	95% CI	Association	
G-A-T-G-G-T-A-T-A-C-A	0.04957	0.04157 0.04957	NS		0.04093	0.02801 0.05385	NS	
A-G-T-G-G-T-A-T-A-C-A	0.04133	0.03400 0.04133	NS		*		NS	
A-G-T-G-G-T-A-T-A-C-G	0.07315	0.06357 0.07315	NS		0.07809	0.06059 0.09559	NS	
G-A-T-A-C-C-G-T-A-C-G	0.39916	0.38113 0.39916	NS		0.43251	0.40020 0.46483	NS	
A-G-T-A-C-C-G-T-A-C-G	0.05503	0.04663 0.05503	AE, OR = 0.26; P = .004		0.05985	0.04438 0.07532	AE, OR = 0.12; P = .003	
G-A-C-A-C-C-G-C-G-T-G	0.17507	0.16107 0.17507	IgE, OR = 0.51; P = .008		0.16114	0.13716 0.18512	NS	
A-G-T-G-G-T-A-T-G-T-G	*	*	NS		0.03044	0.01924 0.04165	NS	

†The relevant SNP positions among the haplotype sequence are indicated by bold letters.

*Frequency < 3%. AE, Atopic eczema.

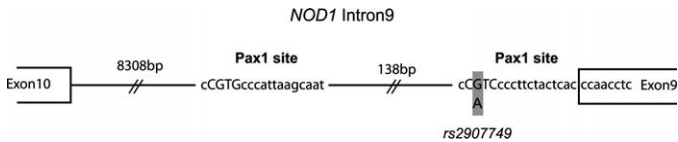


FIG 4. Putative Pax1 binding sites in intron 9 of *NOD1*. The core sequence for Pax1 is presented in *capital letters*.

TABLE IV. Results of population-based and family-based association analyses of the *NOD1* variants and atopic phenotypes*

SNP ID	Atopic eczema		Asthma		IgE†	
	KORA C	Trios	KORA C	Trios	KORA C	Trios
rs2736726	.036	NS	NS	NS	NS	.004
rs2075817	.046	NS	NS	NS	NS	.006
rs2975632	NS	.043	NS	.036	NS	NS
rs3020207	NS	NS	NS	NS	NS	<.0001
rs2075818	NS	NS	NS	NS	NS	.023
rs2235099	NS	NS	NS	NS	NS	.016
rs2075821	NS	NS	NS	NS	NS	NS
rs2075822	NS	.005	NS	.024	.018	.029
rs2907749	NS	.032	NS	.011	.012	NS
rs2907748	NS	.001	NS	.015	.006	NS
rs5743368	NS	NS	NS	NS	NS	.001

*P values are based on χ^2 with 1 degree of freedom.
†Log-transformed total IgE values.

peptidoglycan has been suggested for NOD1.¹³ Peptidoglycan, of which muramic acid is a major component, has been shown to activate innate immune responses via TLR2 and to stimulate the production of TNF- α , IL-1, and IL-6 in human monocytes and of IL-6, IL-8 and β -defensin-2 in human respiratory epithelial cells.^{38,39} In addition, peptidoglycan from *Staphylococcus aureus*, which is frequently observed in atopic eczema lesions,³² has been shown to stimulate the expression of numerous keratinocyte-derived mediators *in vitro*, and mediation via TLR2 has been suggested.⁴⁰ Recently, associations between a polymorphism of the gene encoding TLR2 and asthma⁴¹ as well as another TLR2-SNP with severe atopic eczema⁴² have been reported, and exposure to muramic acid has been shown to be inversely associated with the prevalence of asthmatic symptoms in children.⁴³ However, it has been suggested that TLR2 stimulation by gram-positive cell walls might be caused instead by contaminating lipoteichoic acids in peptidoglycan preparations, and that peptidoglycan sensing is more likely to occur intracellularly through NOD1/NOD2.⁴⁴

The biological response mediated by NOD1 depends on the cellular context, including upregulation of nuclear factor- κ B activation and caspase-9-induced apoptosis.⁴⁵ In addition, NOD1 enhances function of caspase-1, which is an important regulator of inflammatory reactions by proteolytic processing of proinflammatory cytokines, including pro-IL-1 and pro-IL-18.⁴⁶ Thus, it might be speculated whether altered NOD1 function contributes to an altered balance of prosurvival and proapoptotic signal-

ing and to the balance of cytokines on the level of TH2 and TH1 immune responses.

It is noteworthy that SNP rs2907749, which showed replicated significant association with atopy-related traits, is located in intron 9 of the *NOD1* gene, where 2 putative transcription factor binding sites for Pax1 are found. Importantly, rs2907749 alters the computer-predicted Pax1 binding probability next to exon 9 (Fig 4). Pax1 is expressed in fetal and adult thymic epithelial cells and has been shown to be crucial for T-cell development.⁴⁷ So far, little is known about the role of Pax1 in regulating innate immune functions. NOD1 expression could be demonstrated in embryonic and adult thymus,¹⁰ and recognition of microbial molecules by constituents of the innate immune system is known to activate resting antigen-presenting cells (including epithelial cells in the thymus), which in turn influence T-regulatory cell development (see review⁴⁸). Expression analysis and transcription factor binding studies are needed to clarify the role of this *NOD1* polymorphism in this putative interaction.

In conclusion, our data indicate that *NOD1* gene polymorphisms and haplotypic combinations are important determinants of atopy susceptibility. This might be caused by altered interactions with bacterial stimuli, resulting in an inappropriate immunomodulation and/or affection of regulatory processes during lymphopoiesis and immunopoiesis. Further functional studies are necessary to evaluate the functional effect of *NOD1* variants and their meaning in the context of atopic diseases.

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