

Toll-like receptor 2 as a major gene for asthma in children of European farmers

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Background: The finding that the prevalence of asthma and allergies is less frequent in children raised on animal farms has led to the conjecture that exposure to microbial products modifies immune responses. The toll-like receptors (TLRs) represent an evolutionarily conserved family of innate immunity receptors with microbial molecules as ligands.

Objectives: We reasoned that polymorphisms in genes encoding TLRs might modulate the protective effects observed in farming populations.

Methods: Farmers' and nonfarmers' children living in rural areas in Austria and Germany and who were enrolled in the cross-sectional ALEX study were genotyped for single nucleotide polymorphisms in the *TLR2* and *TLR4* genes. The frequencies of asthma, allergic rhinitis, and atopic sensitization were compared between the genotypes in relation to exposure to farming and endotoxin.

Results: Among farmers' children, those carrying a T allele in *TLR2*–16934 compared with children with genotype AA were significantly less likely to have a diagnosis of asthma (3% vs 13%, $P = .012$), current asthma symptoms (3% vs 16%, $P = .004$), atopic sensitization (14% vs 27%, $P = .023$), and current hay fever symptoms (3% vs 14%, $P = .01$). The association between *TLR2*–16934 and asthma among children of farmers was independent of atopy. No such association was found among children from the same rural communities but not living on farms.

Conclusion: Our results suggest that genetic variation in *TLR2* is a major determinant of the susceptibility to asthma and allergies in children of farmers. (*J Allergy Clin Immunol* 2004;113:482-8.)

Key words: Farming, endotoxin, asthma, atopy, children, *TLR2*, *TLR4*, gene-environment interaction

Abbreviations used

EU: Endotoxin units

OR: Odds ratio

TLR: Toll-like receptor

Asthma and allergies are complex diseases with a strong familial component suggestive of a genetic predisposition.^{1,2} There have been numerous reports of an increase in the prevalence of asthma and allergies over the past decades in countries with a high socioeconomic status that cannot be explained by a change of the genetic background. Of interest in this regard are recent studies in Europe and North America pointing to the key role of a strong environmental influence because children and adults raised on animal farms were consistently found to have a lower prevalence of asthma, hay fever, and IgE-mediated reactivity to local allergens than those living away from farms.³⁻⁹ Because a large variety of microbes is detectable on animal farms,¹⁰ these findings are in line with the hypothesis that exposure to microbial products in early life modifies immune responses away from asthma and allergies.^{11,12} In support of this contention, we have recently shown that exposure to bacterial endotoxin was inversely related to the frequency of atopic asthma, hay fever, and sensitization against common aeroallergens in school-aged children.¹³

Many bacteria have pattern molecules on their surfaces, and these molecules interact with pattern-recognition receptors, which are part of the innate immune system. Among the known pattern-recognition receptors for microbial products, toll-like receptors (TLRs) are an evolutionarily conserved group of molecules expressed in antigen-presenting cells and epithelial cells.¹⁴ There are currently 10 different TLRs described in human subjects, and *TLR2* and *TLR4* have been studied most extensively in cell cultures and in animal models. In general, *TLR2* has been found to be part of the signaling complex in response to a wide range of components of the cell membrane of gram-positive and gram-negative bacteria, mycoplasma, mycobacteria, parasites, and yeast,¹⁵ whereas *TLR4* seems to be the exclusive TLR for LPS, a major component of the outer cell membrane of gram-negative bacteria.¹⁶ The genes encoding TLRs show a high variability in human populations,¹⁷ but whether these genetic variations modify the interaction with microbial molecules and thereby modify the frequency of asthma and allergies is not yet known.

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We therefore reasoned that if microbial products are responsible for the lower prevalence of asthma and allergies among farmers' children and in children heavily exposed to endotoxin, then polymorphisms in *TLR2* and *TLR4* might modulate these effects. We tested this hypothesis in children from rural communities in Austria and Germany who were enrolled in the cross-sectional ALEX (Allergy and Endotoxin) study.⁸

METHODS

Population, phenotypes, and exposure to farming and endotoxin

Our study population included 609 children in school grades 1 to 6 from rural areas in Austria and Bavaria (Germany) who took part in the cross-sectional ALEX study, as previously described.⁸ In brief, a self-administered questionnaire on respiratory and allergic diseases was filled out by the parents of children living on a farm and the parents of children living in the same rural area but not raised on a farm. Participating children and their parents were asked for consent to venipuncture and to collect a dust sample from their homes. The analyses in this study were restricted to children from Austria and Bavaria because samples for genotyping from the Swiss children were not available.

Asthma diagnosis was defined as a physician's diagnosis of asthma or recurrent asthmatic, obstructive, or spastic bronchitis. Children whose parents reported "wheezing or whistling in the chest in the last 12 months" were classified as having current asthma symptoms. Current hay fever symptoms were defined as a positive response to the following question: In the last 12 months, has your child had problems with sneezing or a runny or blocked nose without a cold accompanied by itchy-watery eyes? Atopy was defined as a specific IgE level of 3.5 kU/L or greater to at least one of the allergens tested. The concentration of specific IgE against grass pollen, birch pollen, house dust mite (*Dermatophagoides pteronyssinus*), cat dander, cow epithelium, and storage mite (*Lepidoglyphus destructor*) was measured in all sera (CAP, Pharmacia) in a central laboratory (University Children's Hospital Berlin Charity).

Farmers' children were defined as children whose parents answered yes to the following question: Do you live on a farm? For measurement of endotoxin, dust samples were taken from the child's mattress,¹³ stored at room temperature, and shipped to a central laboratory (in Munich, Germany). The content of endotoxin in all dust samples was determined by using a kinetic Limulus assay (Bio Whittaker)¹⁸ and expressed in endotoxin units (EU) per milligram. High endotoxin exposure was defined in children with endotoxin concentrations of greater than the median of the study group and compared with those less than the median.

Genotyping

As part of the National Heart, Lung, and Blood Institute Programs for Genomic Applications, we recently catalogued genetic variation in a central family of pattern-recognition receptors, the *TLR* genes. Details of the resequencing strategy and a full account of the results from 3 ethnic groups (white, African American, and Hispanic subjects), including linkage disequilibrium, main haplotypes found, and genotype frequencies, are available on the Programs for Genomic Applications Web site (<http://innateimmunity.net>).

Single nucleotide polymorphisms needed to identify haplotypes seen more than once in the screening process among white subjects and with a minor allele frequency of 10% or more were genotyped on the basis of the assumption that for polymorphisms with a lower minor allele frequency, power would be less than 0.50 to reject the

hypothesis of no association with an α value of 0.05 (2-sided) in our sample. These single nucleotide polymorphisms were as follows: for *TLR2*, *TLR2*/–16934, *TLR2*/+596, and *TLR2*/+1349; for *TLR4*, *TLR4*/–6143, *TLR4*/–5724, *TLR4*/+7263, and *TLR4*/+8469. In addition, we genotyped the population for *TLR4*/+4434 because it induces a change in the amino acid sequence of the protein, and this polymorphism is associated with hyporesponsiveness to inhaled endotoxin in human volunteers.¹⁹

DNA samples were isolated from whole blood by using standard techniques. DNA concentration was determined by using fluorescence spectroscopy (PicoGreen, Molecular Probes). Genotyping of DNA samples was performed with 5'-exonuclease (Taqman, Applied Biosystems) assays. Each assay was optimized for the PCR annealing temperature. Optimized genotyping reactions (10 μ L of total volume, 0.9 μ mol/L PCR primers, 0.2 μ mol/L of each probe, 20 ng of genomic DNA, 1 $\times$ master mix; Applied Biosystems) were set up in 384-well plates and thermal cycled in MJ tetrads (10 minutes at 95°C; 40 cycles of 15 seconds at 95°C; T_{am} , 1 minute). Taqman reactions were analyzed in a 7900HT (Applied Biosystems) instrument. Each 384-well plate contained 15 samples for which the genotype had been determined independently by means of DNA sequencing, as well as 4 no-DNA blank wells. Accuracy of genotypes (concordance of DNA sequencing derived genotypes to Taqman derived genotypes) was greater than 99.5%.

Oligonucleotide sequences used for genotyping are given in Table I.

Statistical analyses

The frequencies of the polymorphisms in the study population were calculated and compared with a population in Hardy-Weinberg equilibrium by using a χ^2 goodness-of-fit test. The same procedure was done in farmers' and nonfarmers' children separately.

The frequencies of asthma, current asthma symptoms, hay fever, and atopy were compared between the genotypes of the polymorphisms in *TLR2* and in *TLR4* and with respect to exposure to farming and exposure to endotoxin. The difference in proportions was calculated by using the χ^2 test and the Fisher exact test as appropriate. The Mantel-Haenszel common odds ratio (OR) for the association between genotype and phenotype was computed and tested for homogeneity across strata of exposure.

Logistic regression models were used to estimate the effect of the genotype when potential confounding factors were taken into account. The following variables were considered as potential confounders and included in the logistic regression model: study area (Germany/Austria), sex, age in years (continuous), maternal education (low/medium/high), and exposure to tobacco smoke at home (yes/no). We additionally included farming status (yes/no) as a potential confounder when estimating the effect of the polymorphisms in children stratified by endotoxin exposure (high/low) and vice versa.

We calculated the causative fraction, which was defined as the proportion of the disease that would not have occurred had the factor been absent from the population, as suggested by Miettinen,²⁰ and applied the following equation:

$$\text{Causative fraction} = \frac{([OR \text{ associated with the trait} - 1]/OR \text{ associated with the trait}) \times \text{proportion of patients with the trait}}{[OR \text{ associated with the trait} - 1] + \text{proportion of patients with the trait}}$$

Analyses were carried out by chromosomes to test for the association between *TLR2* and *TLR4* haplotypes and the phenotypes under study.²¹

All analyses were performed with Stata/SE 8.0 software (Stata Corp).

This work was approved by the Institutional Review Board of the University of Arizona, by the Human Subject Committees of the

TABLE I. Oligonucleotide sequences used for genotyping

	Probe (VIC)	PCR-forward	
TLR4/-6143	ACTTAGCATACATAATATT	AAGTGCTTGGAGGATATTACAGTAGAACTA	
TLR4/-5724	TTCACCAACACTTATT	GTGATTACCACATTTACAGACCAGAA	
TLR4/+4434	TCGATGGTATTATTG	GGCCTGTGCAATTTGACCAT	
TLR4/+7263	CATCCACTCTTCC	GTTTCCTGTTGGGCAATGCT	
TLR4/+8469	CAAATGCACACATCT	GGTGTTCATGTCTCATGTACTAGTG	
TLR2/-16934	TCTGGTGAGGGTCAT	TGGTTCTGGAGTCTGGGAAGTC	
TLR2/+596	AGATGACTTACATTCTG	CAGATCTACAGAGCTATGAGCCAAAA	
TLR2/+1349	CACGTGTAACAGGC	ATTTGAACCTATCCAGCACACGAAT	
	Probe (FAM)	PCR-reverse	T _{an} (°C)
TLR4/-6143	ACTTAGCATGCATAATA	GGAAAGTAGCAAGTGCAATGTAAGTTT	56
TLR4/-5724	CACCAACGCTTATT	CCACAAATGGTGACAGGAGTTCTC	58
TLR4/+4434	CTCGATGATATTATTG	AGTCACACTCACCAGGGAAAATG	59
TLR4/+7263	AACATCCACTGTTCC	CATTAATCCAGACACATTGTTTCTC	52
TLR4/+8469	AAATGCGCACATCT	CCTGATAGGGATACATAGGGATATGTG	54.5
TLR2/-16934	ATCTGGAGAGGGTCAT	CTCACCATGTGATGCTTTCCAT	60
TLR2/+596	TGACTTACGTCTCTGAATT	CATTCCACGGAACCTGTAAACATCTAC	60
TLR2/+1349	CACAGCGTAACAGG	TCCAGTGTCTTGGGAATGCA	60

FAM, Carboxyfluorescein; T_{an}, annealing temperature.

TABLE II. Frequency of the genotypes in *TLR2* polymorphisms in farmers' children and in nonfarmers' children

	Farming	Nonfarming
TLR2/-16934	n = 237	n = 387
AA	23.2%	25.6%
AT	50.2%	49.9%
TT	26.6%	24.5%
χ ² test	P > .2	
TLR2/+596	n = 237	n = 386
CC	16.9%	18.1%
CT	52.7%	48.7%
TT	30.4%	33.2%
χ ² test	P > .2	
TLR2/+1349	n = 239	n = 386
TT	90.0%	88.1%
CT	10.0%	11.4%
CC	0.0%	0.5%
Fisher exact test	P > .2	

Children's Hospital, by the University of Munich, and by the Children's Hospital, Salzburg, Austria. The nature and possible consequences of the studies were explained to the parents of the children involved, who provided written informed consent for this study.

RESULTS

Our study population included 229 farmers' children and 380 children from the same rural area but not living on a farm. The mean age was 9.4 years (SD, 1.5) in farmers' children and 9.3 years (SD, 1.5) in nonfarmers' children. There were slightly more boys among the farmers' children (56%) than among the nonfarmers' children (51%).

The median of the endotoxin concentrations from the mattress samples was 27.4 EU/mg dust (interquartile range, 24.0 EU/mg) and, as previously reported,¹³ was significantly higher in farmers' children than in nonfarmers' children (37.6 EU/mg [interquartile range, 27.2

EU/mg] and 23.0 EU/mg [interquartile range, 17.5 EU/mg], respectively, $P < .0005$). Sixty-eight percent (156) of the farmers' children and 39% (149) of the nonfarmers' children were classified as heavily exposed to endotoxin (median or greater). In the high-exposure group the median of the endotoxin concentration was 41.3 EU/mg (interquartile range, 21.8 EU/mg), and in the low-exposure group the median of the endotoxin concentration was 17.4 EU/mg (interquartile range, 9.0 EU/mg). The association between endotoxin and asthma, hay fever, and atopy in the ALEX study population are described in detail elsewhere.¹³

The allele frequencies for all single nucleotide polymorphisms were not significantly different between farmers and nonfarmers, and none of them showed marked deviations from a population in Hardy-Weinberg equilibrium (Tables II and III). No difference in exposure to endotoxin was found between the genotypes of the polymorphisms in *TLR2* and *TLR4*.

TLR2 polymorphisms

Among farmers' children there was a strong association between the *TLR2*/-16934 polymorphism and the phenotypes under study. Only those children carrying 1 or 2 T alleles in *TLR2*/-16934 were significantly less likely to have a diagnosis of asthma, current asthma symptoms, atopy, and current hay fever symptoms (Fig 1, A). No such association was found in nonfarmers' children (Fig 1, B). The ORs for the association between the *TLR2*/-16934 polymorphism and the phenotype were different between farmers' and nonfarmers' children for asthma (test for homogeneity for the ORs, $P = .07$), current asthma symptoms ($P = .008$), atopy ($P = .005$), and current hay fever symptoms ($P = .007$).

Logistic regressions were performed to determine the potential role of confounders in these results. For *TLR2*/-16934, associations remained significant for all

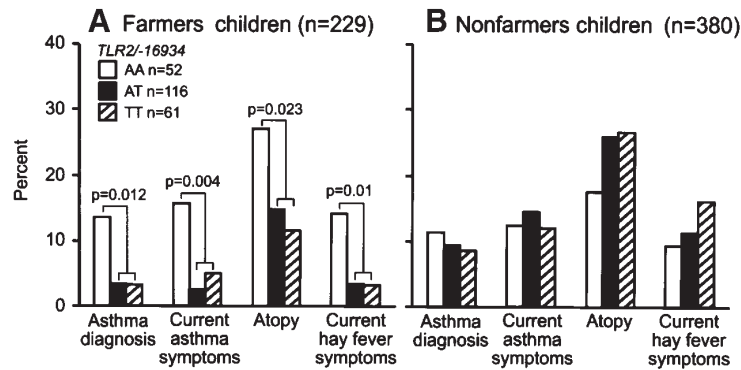


FIG 1. Prevalence of asthma, atopy, and current hay fever symptoms in *TLR2*/-16934 in farmers' children (A) and in nonfarmers' children (B).

phenotypes under study in the case of farmers and remained nonsignificant for the same phenotypes among nonfarmers (Table IV). When, in models with asthma as a dependent variable, specific IgE against any of the allergens tested was also included in the model together with the other confounders, the association remained significant for asthma diagnosis (OR, 0.18; 95% CI, 0.04-0.79; $P = .02$) and for current asthma symptoms (OR, 0.16; 95% CI, 0.05-0.58; $P = .005$). In farmers the causative fraction that is attributable to the genotype AA in *TLR2*/-16934 was 44% for asthma diagnosis, 47% for current asthma symptoms, 21% for atopy, and 41% for current hay fever symptoms.

We next assessed the association between *TLR2*/-16934 and asthma and allergies separately for subjects heavily exposed (≥ 50 th percentile) and for those not heavily exposed to endotoxin. No clear association between genotypes and phenotypes was observed for either subjects heavily exposed to endotoxin or for subjects not heavily exposed (Table IV).

We found no association between either *TLR2*/+596 or *TLR2*/+1349 and any of the phenotypes under study for either farmers or nonfarmers or in relationship to endotoxin, and no additional information was obtained by using *TLR2* haplotypes (data not shown).

TLR4 polymorphisms

There was no statistically significant association between the *TLR4*/+4434 polymorphisms and either asthma, allergic rhinitis, or atopy when the data were stratified by farming status (Table IV). However, when the data were stratified by level of exposure to endotoxin, we found that among children who were heavily exposed, *TLR4*/+4434 showed a significant association with atopy (Fig 2, A), and a trend in the opposite direction was seen in children not heavily exposed (Fig 2, B). The Mantel-Haenszel test for homogeneity for the ORs across strata of endotoxin exposure was highly significant ($P = .014$). The association between the polymorphism and atopy remained significant after controlling for potential confounders (Table IV). No clear association was seen for asthma, current asthma symptoms, and current hay fever symptoms (Fig 2 and Table IV). Very

TABLE III. Frequency of the genotypes in *TLR4* polymorphisms in farmers' children and in nonfarmers' children

	Farming	Nonfarming
<i>TLR4</i> /-6143	n = 231	n = 385
AA	46.3%	44.9%
AG	41.1%	45.7%
GG	12.6%	9.4%
χ^2 test	$P > .2$	
<i>TLR4</i> /-5724	n = 240	n = 396
TT	71.3%	75.5%
CT	25.4%	22.0%
CC	3.3%	2.5%
χ^2 test	$P > .2$	
<i>TLR4</i> /+4434	n = 236	n = 395
AA	88.1%	85.1%
AG	11.9%	14.2%
GG	0.0%	0.8%
Fisher exact test	$P > .2$	
<i>TLR4</i> /+7263	n = 241	n = 395
GG	71.0%	72.9%
CG	26.6%	26.3%
CC	2.5%	0.8%
Fisher exact test	$P > .2$	
<i>TLR4</i> /+8469	n = 242	n = 395
TT	76.4%	74.9%
CT	20.2%	23.3%
CC	3.3%	1.8%
χ^2 test	$P > .2$	

similar results were obtained when endotoxin exposure was assessed as a continuous variable (data not shown).

No associations were seen for the other *TLR4* polymorphisms, and no additional information was obtained with *TLR4* haplotypes (data not shown). No significant gene-gene interaction was found between the *TLR4* and *TLR2* single nucleotide polymorphisms (data not shown).

DISCUSSION

In this study we found that a polymorphism in the *TLR2* gene is strongly associated with the frequency of asthma and allergies in children of European farmers and that a

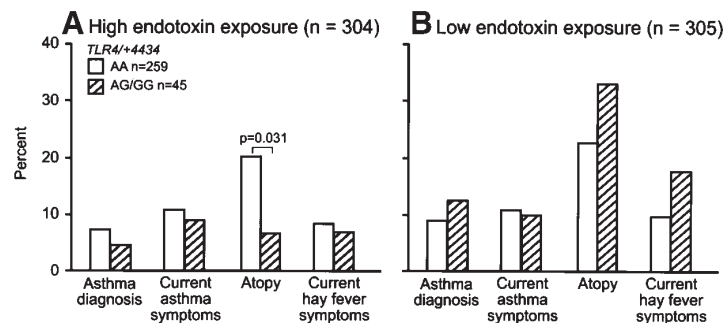


FIG 2. Prevalence of asthma, atopy, and current hay fever symptoms in *TLR4*/+4434 in children exposed to high (≥ 50 th percentile; **A**) and children exposed to low (< 50 th percentile; **B**) endotoxin concentrations (in EUs per milligram).

TABLE IV. ORs, adjusted ORs,* and 95% CIs for asthma, atopy, and current hay fever symptoms in farmers' and in nonfarmers' children and in children exposed to high (≥ 50 th percentile) and low (< 50 th percentile) endotoxin concentrations (in EUs per milligram) in relation to *TLR2*-16934 (AA, n = 149; AT/TT, n = 460) and *TLR4*/+4434 (AA, n = 525; AG/GG, n = 84) polymorphisms

	Farmers (n = 229)		Nonfarmers (n = 380)	
	OR (95% CI)	aOR† (95% CI)	OR (95% CI)	aOR† (95% CI)
<i>TLR2</i> -16934				
Asthma diagnosis	0.23 (0.07-0.70)	0.17 (0.04-0.77)	0.79 (0.38-1.67)	0.71 (0.33-1.53)
Current asthma symptoms	0.19 (0.06-0.57)	0.18 (0.05-0.61)	1.12 (0.56-2.24)	1.07 (0.53-2.17)
Atopy	0.43 (0.20-0.90)	0.41 (0.18-0.91)	1.67 (0.93-3.00)	1.60 (0.86-2.98)
Current hay fever symptoms	0.22 (0.07-0.67)	0.24 (0.07-0.84)	1.42 (0.66-3.07)	1.47 (0.65-3.33)
<i>TLR4</i> /+4434				
Asthma diagnosis	0.67 (0.08-5.36)	1.73 (0.17-17.19)	1.06 (0.42-2.66)	1.09 (0.43-2.77)
Current asthma symptoms	§		1.03 (0.46-2.32)	1.07 (0.47-2.46)
Atopy	0.19 (0.02-1.43)	0.22 (0.03-1.72)	1.10 (0.58-2.08)	1.09 (0.55-2.16)
Current hay fever symptoms	0.66 (0.08-5.30)	0.96 (0.11-8.43)	1.42 (0.65-3.14)	1.29 (0.56-2.97)
	Endotoxin: high (n = 304)		Endotoxin: low (n = 305)	
	OR (95% CI)	aOR† (95% CI)	OR (95% CI)	aOR† (95% CI)
<i>TLR2</i> -16934				
Asthma diagnosis	0.61 (0.23-1.56)	0.72 (0.26-2.01)	0.51 (0.23-1.13)	0.42 (0.18-1.00)
Current asthma symptoms	0.67 (0.30-1.48)	0.72 (0.31-1.71)	0.70 (0.32-1.56)	0.70 (0.30-1.63)
Atopy	0.65 (0.34-1.23)	0.72 (0.37-1.43)	1.58 (0.83-3.03)	1.46 (0.73-2.93)
Current hay fever symptoms	0.63 (0.26-1.53)	0.93 (0.34-2.53)	1.03 (0.44-2.38)	0.89 (0.36-2.19)
<i>TLR4</i> /+4434				
Asthma diagnosis	0.59 (0.13-2.62)	0.54 (0.12-2.51)	1.48 (0.53-4.13)	1.46 (0.50-4.27)
Current asthma symptoms	0.80 (0.27-2.41)	0.76 (0.24-2.39)	0.96 (0.32-2.89)	0.89 (0.29-2.78)
Atopy	0.29 (0.09-0.96)	0.17 (0.04-0.75)	1.67 (0.81-3.45)	1.93 (0.89-4.19)
Current hay fever symptoms	0.78 (0.22-2.72)	0.55 (0.12-2.57)	1.98 (0.79-4.92)	2.06 (0.79-5.37)

aOR, Adjusted OR.

*Logistic regression was used to adjust for study area, sex, age, maternal education, and exposure to tobacco smoke at home; reference genotype was AA for *TLR2* and AA for *TLR4*, respectively.

†Additionally adjusted for current endotoxin exposure.

‡Additionally adjusted for farming.

§No child with current asthma symptoms in genotype AG/GG.

polymorphism in the *TLR4* gene has an influence on atopy in children heavily exposed to endotoxin. These results point to a gene-by-environment interaction because both *TLR2* and *TLR4* are genes encoding ancient pattern-recognition receptors for different microbial molecules.

The potential role of TLRs as regulators of immune responses has received considerable attention in recent

years, and TLR activation in different types of cells is associated with the release of effector molecules, such as cytokines and chemokines,²² that can influence adaptive immune responses. TLRs are present on the surfaces of antigen-presenting cells, regulatory T cells, and other cells that play crucial roles in the interface between environmental exposures and the immune system.^{23,24} Sig-

nals produced by these cells control the maturation of both CD4⁺ and CD8⁺ T cells, and this maturation determines the type of adaptive immune response against environmental antigens. It was thus plausible to surmise that genetic variations in TLRs might have an influence in the pattern of immune responses that are established against environmental antigens.

In our study we found that a genetic variation in *TLR2* (*TLR2*/–16934) is a major determinant of susceptibility to asthma and atopy in farmers' children. That microbial products present in a farming environment might interact with TLR2 is supported by the finding of an increased expression of this receptor in blood cells obtained from children of farmers compared with children not raised on farms.²⁵ It is not possible to deduce from our study the molecular mechanisms involved in the interaction between polymorphisms in *TLR2* and exposures present in children raised on farms because the functional significance of this polymorphism is not known. Moreover, other polymorphisms that are in linkage disequilibrium with *TLR2*/–16934 could be responsible for the observed associations. However, if *TLR2*/–16934 were the polymorphism mainly responsible for the observed effects, carriers of the T allele could be less likely to have asthma and allergies as a result of an increased expression of TLR2 on the surface of antigen-presenting cells or other cells. This, in turn, could increase their susceptibility to the protective effects of TLR2 ligands present in microbial products. It is not known whether a specific microbial molecule is essential for the observed association between asthma and the *TLR2* polymorphism in farmers' children or if activation of a common intracellular signaling pathway by any of the TLR2 ligands is sufficient. TLR2 interacts with components of a variety of gram-positive and gram-negative bacteria, mycobacteria, yeast, and parasites,^{26–29} which are likely to be abundant in an animal-farming environment, but the design of this study does not allow us to identify the specific pattern-recognition molecules underlying our observed association.

In this study we also found that a genetic variation in *TLR4*/+4434 is inversely associated with specific IgE to common aeroallergens in children heavily exposed to endotoxin. This is of interest because TLR4 is the exclusive TLR for LPS, the purified endotoxin from gram-negative bacteria, and *TLR4*/+4434 leads to an amino acid change that alters the extracellular domain of the TLR4 receptor.¹⁹ In recent studies, carrying the minor G allele for *TLR4*/+4434 was associated with bronchial hyporesponsiveness to inhaled LPS in healthy volunteers.¹⁹ In German adults only subjects with the minor G allele in *TLR4*/+4434 showed an inverse association between exposure to house dust endotoxin and bronchial responsiveness to methacholine.³⁰ In our study population we previously found that exposure to indoor endotoxin has protective effects against the development of atopy and atopy-related phenotypes,¹³ and we speculate that responsiveness to endotoxin in carriers of the minor G allele in *TLR4*/+4434 explains, at least in part, the observed inverse association. Despite a strong associa-

tion between the *TLR4*/+4434 polymorphism and specific IgE in subjects heavily exposed to endotoxin, no clear association was seen between *TLR4*/+4434 and either asthma or hay fever in these same subjects. A limited number of carriers of the minor G allele in *TLR4*/+4434 in our data set could be one explanation for this discrepancy. Nevertheless, the role in the genetics of asthma and allergies of variations in other molecules (apart from TLR4) involved in the endotoxin receptor and signaling mechanisms is only beginning to be understood.³¹

The results of our study further suggest that the genetic and environmental mechanisms that determine asthma might be different from those determining atopy, despite a strong association between these 2 conditions in the population as a whole. Indoor endotoxin appears to modify the production of specific IgE and, by this mechanism, has a protective effect against hay fever and the atopic form of asthma, but it does not seem to have a direct protective effect against the development of nonatopic asthma.¹³ On the other hand, a farming environment is associated with a lower prevalence of both atopic and nonatopic asthma,⁸ suggesting that microbial products other than endotoxin play an important role in the association between exposure to animal farms and asthma. Our finding of an association between the *TLR2*/–16934 polymorphism and asthma independent of atopy in farmers' children supports this conclusion.

The observed associations in farmers' children are unlikely due to population stratification. We found the associations in Austrian and in German farmers' children, and in both countries there were no such associations among children of nonfarmers. However, replication of our results in an independent study is clearly needed. We cannot exclude the possibility, for example, that avoidance of exposure to TLR2 ligands and other microbial products might have occurred in homozygotes for the A allele in *TLR2*/–16934, thus making them more susceptible to asthma and allergies among farmers' children. Further studies are needed to identify the TLR2 ligands responsible for the observed association to exclude the possibility that preferential avoidance of certain exposures might explain our findings.

Identification of products present in the environment that are responsible for the decreased risk of asthma in farmers' children might prove useful for future strategies for the primary prevention of asthma. Exposure to innocuous surrogates of these molecules at crucial times during the development of asthma-related immune responses in susceptible individuals could be associated with a marked decrease in the incidence of these diseases in much the same way as natural exposure to microbial products among children of farmers.

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