Genetic polymorphisms in pattern recognition receptors are associated with allergic diseases through gene–gene interactions

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Abstract

Background. There is evidence that suggests variation in gene encoding pattern recognition receptors, the essential components of innate immunity, might be associated with atopic diseases. However, results have been inconclusive.

Objectives. The aim of the study was to determine the individual associations and possible interactive effects of the *CD14* (cluster of differentiation 14), *TLR4* (toll-like receptor 4) and *TLR2* (toll-like receptor 2) polymorphisms on allergic diseases.

Material and methods. The *CD14 C-159T*, *TLR4 +896A/G* and *TLR2 A-16934T* polymorphisms were identified in 115 children aged from 6 to 17 years. All subjects were selected using a detailed questionnaire which included questions on symptoms and each one underwent skin prick testing. All single-nucleotide polymorphisms (SNPs) were determined using real-time polymerase chain reaction (PCR) assays.

Results. There was no statistically significant correlation between the 3 polymorphisms (*CD14 C-159T*, *TLR4 +896A/G* and *TLR2/-16934A/T*) and either asthma, allergic rhinitis or atopy. We observed that children who were heterozygous or homozygous for both the *CD14/-159T* and *TLR2/-16934A* alleles had a 4-fold lower risk for asthma than children who were carriers of the T allele of *CD14* but non-carriers of the A allele of *TLR2*, and an almost 3-fold lower risk for asthma when compared to all other groups. Concerning allergic rhinitis, a similar trend was observed. In addition, the presence of at least 1 A allele in the *TLR2/-16934* polymorphism reduced the risk for asthma and allergic rhinitis, but only in children who were homozygous for the common A allele in the *TLR4 +896* polymorphism.

Conclusions. Our study supports the idea that the *CD14*, *TLR2* and *TLR4* polymorphisms may not be directly involved in the development of atopic diseases. However, our results suggest that their impact on the risk of asthma and allergic rhinitis might be modulated by gene–gene interactions.

Key words: single nucleotide polymorphisms, asthma, innate immunity, atopy, gene–gene interactions

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Introduction

Toll-like receptors (TLRs) and cluster of differentiation 14 (CD14) are pattern recognition receptors (PRRs) that are critically involved in activating innate immunity and in inducing subsequent adaptive immune responses. These receptors are expressed in a wide variety of innate immune cells, including epithelial cells, macrophages, mast cells, eosinophils, and dendritic cells. Toll-like receptors and CD14 recognize and respond to diverse microbial molecules that are often referred to as pathogen-associated molecular patterns (PAMPs).[1,2] Experimental studies have largely implicated TLRs and CD14 in both the development and control of the allergic reaction.[3,4] The role of these receptors in the pathogenesis of allergic diseases results from the biological role that they play in activating and regulating the immune response.[2,4] Toll-like receptors and CD14 stimulation via microbial products activates antigen-presenting cells, influences the function of T regulatory cells (T_{reg}), determines the Th_1/Th_2 balance and Th_{17} cell differentiation, and controls cytokine production in mast cells and eosinophil activation.[2,4,5] The discovery of TLRs and their signaling pathways provides an immunological basis for the hygiene hypothesis, which suggests that allergic diseases develop because of changes in microbial exposure and PPR stimulation early in life.[6,7]

Because toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4) and CD14 are so centrally involved in the recognition of the microbial environment, it has been speculated that genetic changes in these molecules may modulate responsiveness to lipopolysaccharides (LPSs) and other PAMPs, leading to inadequate immune responses and thus playing a role in the development of atopy and other allergic diseases.[8] There is growing evidence coming from functional studies that polymorphisms in genes which encode TLR2, TLR4 and CD14 significantly influence transcriptional activity, gene expression and/or receptor function, making them biologically plausible candidate genes for asthma and allergic diseases.[9–11]

TLR2 is a key component in linking microbial products, apoptosis and host defense mechanisms. It identifies elements of the cell walls of gram-positive and gram-negative bacteria, protein components of viruses, and molecules of bacillus, fungi and parasites.[3,4,12] The *TLR2/-16934A/T* polymorphism is located in a region that significantly influences the process of transcription of the *TLR2* gene. A much higher level of transcription of the TLR2 receptor has been demonstrated on monocytes of healthy volunteers with the homozygotic genotype TT, in comparison with monocytes of the wild allele A carriers.[9]

TLR4 is the principal receptor for recognizing LPSs – the main component of the cell walls of gram-negative bacteria – and it plays a critical role in the innate immune response to both gram-negative pathogens and respiratory syncytial virus.[3,4,12] Arbour et al. confirmed that the presence of the *TLR4/+896A/G* polymorphism is associated with hyporesponsiveness to inhaled endotoxin, reduced density of TLR4 in airway epithelium and reduced production of inflammatory cytokines in response to endotoxins.[10] Another experimental study showed that the *TLR4/+896A/G* polymorphism is associated with a blunted systemic inflammatory response to inhaled lipopolysaccharides.[13]

The *CD14* gene encodes a protein that functions as a co-receptor for TLR4 and is one of the major components in the innate immunity response. The CD14 receptor is multifunctional with a high specificity for LPSs, and together with TLR4 forms the complex that activates the innate immune system through various pathways.[1,14] The *CD14* gene is located on chromosome 5q31.3, a region associated with asthma in a genome-wide linkage study.[15] Functional research has shown that the *CD14/-159C/T* polymorphism significantly influences the intensity of transcription and expression of the gene.[11] This data is reflected in numerous clinical studies confirming that the *CD14/-159C/T* polymorphism is connected with a higher concentration of sCD14 in blood serum and a lower concentration of total immunoglobulin E (IgE).[16–21] Based on these results of functional studies, it was hypothesized that polymorphisms within *TLR2*, *TLR4* and *CD14* genes – by changing the gene expression and/or the function of the receptor – may modify the immunological response towards microbiological factors and influence the risk of allergy development. Numerous studies have implicated polymorphisms of the *TLR2*, *TLR4* and *CD14* genes in the pathogenesis of asthma and allergies. Among the reported studies, most found that these polymorphisms were associated with asthma and allergic diseases, though the reports are contradictory.[22–25] In the ALEX study, the wild-type *TLR2/-16934T* allele was found to be associated with fewer asthma diagnoses, symptoms and atopy among German and Austrian farmers' children, but this relationship was not observed in children who did not live on a farm.[26] The *TLR4/+896A/G* variant was found to be associated with 4-fold higher prevalence of asthma and a 7-fold higher prevalence of atopic asthma in a population of Swedish children.[27] Another study found that asthmatic people with the *TLR4/+896A/G* polymorphism have more severe atopy.[28] However, subsequent studies on *TLR4* polymorphisms as a risk factor for atopy and asthma in adults or children have reported conflicting results.[20,26,27,29] Some studies found the *CD14/-159T* allele was associated with lower IgE levels and/or a reduced risk of atopy,[17,18] while other studies found no association[19,30] or an increased risk of atopy and higher IgE levels in subjects with the *CD14/-159T* allele.[31–33] A large meta-analysis of the *CD14/-159C/T* promoter polymorphism and atopic asthma found a significant protective effect for carriers of the TT and CT genotype as compared with the CC genotype.[25] On the other hand, a recent review concluded that there is still no convincing evidence of *CD14* and *TLRs* polymorphisms playing a role in childhood asthma among

Caucasians.[22] Since the results of available published association studies are heterogeneous, further population studies are warranted. Moreover, in the case of complex diseases, the predisposition might be better explained by a combined effect of the variations in multiple genes within the same functional pathway.

Therefore, the aim of this case-control study was to determine the individual associations and possible interactive effect of the *CD14/-159C/T*, *TLR4/+896A/G* and *TLR2/-16934A/T* polymorphisms with respect to atopy, asthma and allergic rhinitis in a population of Polish children

Material and methods

Study population

A total of 115 unrelated children (57 males and 58 females) ranging in age from 6 to 17 years (mean age: 11.8 years) were enrolled in the study. All study participants were of Caucasian ethnicity. The study subjects were recruited from patients who had visited the Outpatient Allergy Clinic for Children in Wrocław, Poland, and from the general population. The healthy control subjects were recruited from the general population through community-based approaches. We distributed flyers at local schools, pediatricians' and family doctors' offices, and during health fairs. Interested parents were instructed to contact the research coordinator via telephone. All participants, case subjects and controls were selected with the help of a detailed questionnaire that included questions regarding overall health status, symptoms of asthma and allergic rhinitis, sociodemographic information, and family history of allergic diseases.

All subjects, including the positive and negative controls, underwent skin prick testing (SPT) for a panel of 6 common inhalant allergens: house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), cat dander, grass pollen (a mix of 6 species), tree pollen I (a mix of 5 species), and tree pollen II (a mix of 4 species) (Allergopharma, Reinbek, Germany). The concentration of total serum IgE was measured using the commercially available IMMULITE 2000 Total IgE kit (Diagnostic Products Corporation, Los Angeles, USA).

Diagnoses of atopy and its related diseases were made according to the following criteria:

Atopy was defined as a positive skin prick test reaction – determined with a wheal 3 mm in diameter – to 1 or more of the 6 common inhalant allergens. Additionally, atopic patients had had symptoms of asthma and/or allergic rhinitis during the previous 12 months.

Asthma was defined using the questionnaire answers along with a physician's diagnosis of asthma, according to the Global Initiative For Asthma (GINA) criteria.[34] Asthma was confirmed by a history of coughing, wheezing and chest tightness during the previous 12 months as well as by positive results of the reversibility tests: >12% reversibility of FEV_1 after β_2-agonist inhalation.

Diagnoses of allergic rhinitis were based on reports of a doctor's diagnosis of allergic rhinitis and/or the occurrence of 2 or more symptoms (nasal obstruction, rhinorrhea, sneezing, or itchy nose) in the absence of a common cold during the previous 12 months, plus sensitization against common inhalant allergens.

The control group, matched with the case group for age and gender, included healthy children who met the following criteria: an absence of symptoms of asthma, allergic rhinitis or other allergic diseases, a negative SPT result, and a negative family history of allergic diseases.

Genotyping

The samples of the 115 subjects were genotyped for the *CD14 C-159T*, *TLR4 +896 A/G* and *TLR2 A-16934T* polymorphisms. Genomic DNA was obtained from ethylenediaminetetraacetic acid (EDTA) whole blood samples using a QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). All SNPs were determined with real-time polymerase chain reaction (PCR) assays with subsequent melting curve analysis using SimpleProbe® probes (TibMolbiol, Berlin, Germany) which were complementary to the wild-type sequences. Initial PCR amplifications and melting analyses were carried out with various oligonucleotide concentrations and ratios. Melting peaks were optimized when asymmetric PCR conditions (unequal amounts of primers) were used. The reactions were performed on a Light Cycler 1.5 platform (Roche Applied Science, Mannheim, Germany) as described in Table 1. For quality control of the genotyping procedures, positive and negative controls for each genotype were included in each reaction.

Statistical analysis

Hardy–Weinberg equilibrium was tested for using the χ^2 goodness-of-fit test to compare the observed genotype frequencies with the expected frequencies among the controls. Differences in genotype frequencies or demographic characteristics between the case and control groups were evaluated using the χ^2 test or the Fisher's exact test, as appropriate. Correlations of genotypes or alleles with patient groups vs control subjects without atopic diseases were determined by computing the odds ratio (OR), its 95% confidence interval (95% CI) and p-values using logistic regression analysis for crude ORs (and adjusted ORs when adjusting for age and gender). Three different genetic models were tested separately in the comparison of genotypes and atopic diseases in this study. A χ^2 test or Fisher's exact test was used to determine the combined effect of genotype pairs. Gene–gene interaction was investigated using logistic regression models for atopy, asthma and allergic

Table 1. Primer and probe sequences used for genotyping SNPs in the *CD14*, *TLR4* and *TLR2* genes and PCR conditions

Polymorphism	SNP ID	Specific primers	Probes
CD14/-159C/T	rs 2569190	f:5′- CTTCggCTgCCTCTgACAgTT r:5′- ggTgCCAACAgATgAggTTCAC	5′- TTCCTgTXITACggCCCCCCT--PH
Reaction mix		2.5 µL of DNA at a concentration of 15–60 ng/µL, 1.0 µL forward primer (5 µmol/L), 1.0 µL reverse primer (2.5 µmol/L), 1.0 µL probe (2 µmol/L), 2.0 µL of LightCycler®TaqMan®Master	
PCR		12 min 95°C – 55 cycles (10 s 95°C – 20 s 55°C – 20 s 72°C), ramping rate 20°C/s – 45–90°C with 0.1°C/s	
TLR4/+896A/G	rs 4696480	f:5′- AACAgAAATTTATCCATTCATggTT r:5′- AgCAgTTTATTgTgAgAATgAgTTT	5′- CCAgAXITgACCCTCACCAgATgC--PH
Reaction mix		2.5 µL of DNA at a concentration of 15–60 ng/µL, 1.0 µL forward primer (5 µmol/L), 1.0 µL reverse primer (5 µmol/L), 1.0 µL probe (2 µmol/L), 2.0 µL of LightCycler®TaqMan®Master	
PCR		12 min 95°C – 50 cycles (10 s 95°C – 20 s 55°C – 20 s 72°C), ramping rate 20°C/s – 40–80°C with 0.1°C/s	
TLR2/-16934A/T	rs 4986790	f:5′- CCAAgAAgTTTgAACTCATggTAA r:5′- ATTTAAAgAAATTAggCTTCATAAgCT	5′- ACTACCTCgATgATATTATXITgACTTATT--PH
Reaction mix		2.5 µL of DNA at concentration of 15–60 ng/µL, 1.0 µL forward primer (5 µmol/L), 1.0 µL reverse primer (1 µmol/L), 1.0 µL probe (2 µmol/L), 2.0 µL of LightCycler®TaqMan®Master	
PCR		12 min 95°C – 50 cycles (10 s 95°C – 20 s 55°C – 20 s 72°C), ramping rate 20°C/s – 40–90°C with 0.1°C/s	

PCR – polymerase chain reaction; SNP – single nucleotide polymorphism; TLR – toll-like receptor.

rhinitis with interaction terms. Both parametric (t-test and analysis of variance – ANOVA) and non-parametric (Mann–Whitney U test and Kruskal–Wallis test) analyses were performed to study the phenotypic differences in each genotype group, depending on the normality of the distribution of the variables. Statistical significance was set at a p-value <0.05. The statistical analyses were carried out using STATISTICA v. 9.0 (StatSoft, Inc., Tulsa, USA) and the SPSS Statistics v. 11.1 (SPSS Inc., Chicago, USA). A p-value <0.05 was considered statistically significant and all statistical tests were 2-sided.

The study was approved by the Ethics Committee of the Wroclaw Medical University and informed written consent – including consent for genetic studies – was obtained from all of the subjects prior to testing.

Table 2. Characteristics of the study groups. Polysensitization is a positive skin prick tests against at least 3 allergens. Descriptive data is presented as observation count, percent, mean, and standard deviation or median and range

Variable	Cases (n = 67)	Controls (n = 48)
Age [years]	12.4 ±3	11.0 ±2.7
Gender [male/female]	38/29	19/29
Atopy Asthma Allergic rhinitis	67 (100.0) 33 (49.2) 59 (88.0)	0 0 0
Number of positive skin tests	3 (1–6)	0
Polysensitization	43 (64.2)	0
Atopic hereditary Asthma Allergic rhinitis	37 (55.2) 18 (26.8) 26 (38.8)	0 0 0
Total serum IgE [IU/mL]	603 ±801	0

IgE – immunoglobulin E.

Results

The baseline characteristics of the study population are presented in Table 2. The allele and genotype distributions for the *CD14/-159C/T*, *TLR4+896A/G* and *TLR2/-16934A/T* polymorphisms among the case subjects and controls are shown in Table 3. Because the GG genotype of the *TLR4/+896A/G* was rare, we combined the AG and GG genotypes for *TLR4* when calculating p-values. The genotype frequencies of the 3 SNPs studied were in agreement with Hardy–Weinberg equilibrium in all groups, as indicated by the χ^2 values at p-values >0.05 shown in Table 4.

No significant differences in genotype or allelic frequencies for the *CD14/-159C/T*, *TLR4/+896A/G* and *TLR2/-16934A/T* polymorphisms could be observed when the children with atopy, asthma or allergic rhinitis were compared to non-atopic controls. Furthermore, there were no significant correlations between the *CD14/-159C/T*, *TLR4/+896A/G* and *TLR2/-16934A/T* alleles and/or genotypes and total IgE level or the number of positive skin prick tests in all studied groups (data not shown).

Next, we examined if there was a connection between the studied phenotypes and coinheritance of the studied polymorphisms. We observed that children who were heterozygous or homozygous for both the *CD14/-159T* and *TLR2/-16934A* alleles had a 4-fold lower risk for asthma than children who were carriers of the T allele of *CD14* but non-carriers of the A allele of *TRL2* and an almost 3-fold lower risk for asthma when compared to all other groups. Concerning allergic rhinitis, a similar trend was observed: a 3-fold lower risk of having nasal allergies was seen in carriers of the A allele of *TRL2* who were also carriers of the T allele of the *CD14* gene when compared

Table 3. Genotype and allele frequencies of CD14, TLR4 and TLR2 polymorphisms in the study groups. To study a co-dominant model, the 3 genotype groups were analyzed separately using wild-type homozygotes as a reference group. To test a dominant model, wild-type homozygotes were compared with heterozygotes and homozygotes for minor alleles. In a multiplicative model, genotypes were coded as a 3-level variable for the number of minor alleles

Genotype	Control n [%]	Atopy			Asthma			Allergic rhinitis		
		n [%]	p-value	OR (95% CI)	n [%]	p-value	OR (95% CI)	n [%]	p-value	OR (95% CI)
CD14/–159C/T										
CC	13 (27.1)	20 (29.8)	0.945	1.00 (ref)	10 (30.3)	0.848	1.00 (ref)	17 (28.8)	0.832	1.00 (ref)
CT	22 (45.8)	30 (44.8)		0.90 (0.35÷2.34)	13 (39.4)		0.70 (0.22÷2.21)	29 (4.2)		1.09 (0.41÷2.93)
TT	13 (27.1)	17 (25.4)		1.04 (0.35÷3.10)	10 (30.3)		0.87 (0.24÷3.20)	13 (22.0)		1.03 (0.32÷3.33)
CT + TT	35 (72.9)	47 (70.2)	0.461	0.94 (0.39÷2.25)	23 (69.7)	0.948	0.75 (0.26÷2.15)	42 (71.2)	0.100	1.05 (0.43÷2.60)
T	48 (50.0)	64 (47.8)	0.841	1.00 (0.59÷1.72)	33 (50.0)	0.873	0.94 (0.50÷1.76)	55 (46.6)	0.722	0.99 (0.55÷1.92)
TLR4/+896A/G										
AA	44 (91.7)	59 (88.1)	0.758	1.00 (ref)	28 (84.8)	0.475	1.00 (ref)	51 (86.4)	0.541	1.00 (ref)
AG + GG	4 (8.3)	8 (11.9)		1.81 (0.47÷6.93)	5 (15.2)		2.15 (0.49÷9.39)	8 (13.6)		2.13 (0.55÷8.24)
G	4 (4.2)	9 (6.7)	0.591	1.94 (0.57÷6.54)	6 (9.1)	0.319	2.40 (0.66÷8.65)	9 (7.6)	0.392	2.23 (0.65÷7.65)
TLR2/–16934A/T										
TT	11 (22.9)	26 (38.8)	0.184	1.00 (ref)	14 (42.4)	0.158	1.00 (ref)	23 (39.0)	0.206	1.00 (ref)
TA	21 (43.8)	25 (37.3)		0.44 (0.16÷1.17)	12 (36.4)		0.35 (0.10÷1.14)	22 (37.3)		0.49 (0.18÷1.35)
AA	16 (33.3)	16 (23.9)		0.38 (0.13÷1.08)	7 (21.2)		0.31 (0.08÷1.12)	14 (23.7)		0.39 (0.13÷1.17)
TA + AA	37 (77.1)	41 (61.2)	0.110	0.46 (0.19÷1.10)	19 (57.6)	0.105	0.40 (0.15÷1.08)	36 (61.1)	0.117	0.44 (0.18÷1.10)
A	43 (44.8)	57 (42.5)	0.116	0.64 (0.38÷1.06)	26 (39.4)	0.070	0.58 (0.31÷1.10)	50 (42.4)	0.083	0.61 (0.35÷1.05)

ref – reference; TLR – toll-like receptor; OR – odds ratio; CI – confidence interval.

Table 4. Hardy–Weinberg equilibrium values in the study groups. χ^2 test or Fisher's exact test*, p > 0.05

Genotype	Control	Atopy	Asthma	Allergic rhinitis
CD14/–159C/T	p = 0.563	p = 0.400	p = 0.223	p = 0.924
TLR2/16934A/T	p = 0.424	p = 0.053	p = 0.170	p = 0.069
TLR4/+896A/G	p = 1.000*	p = 0.250*	p = 0.219*	p = 0.280*

TLR – toll-like receptor.

to the reference group and to all other genotype combinations. In addition, the presence of at least 1 A allele at the *TLR2/-16934* polymorphism significantly correlated with a decreased risk for asthma and allergic rhinitis, but only in children who were homozygous for the common A allele at the *TLR4/+896* polymorphism. No significant correlation of the *TLR2/-16934A/T* with either asthma or allergic rhinitis was seen in the cases with at least one G allele at the *TLR4/+896* polymorphism. As shown in Table 5, the odds ratios (ORs) and p-values for asthma and allergic rhinitis suggest the presence of a gene–gene interaction, though when modeling for interactions, the interaction coefficient was not significant for either genotype combination (p-value for interaction >0.05). No significant difference in the prevalence of asthma or allergic rhinitis was detected for the combination of the *CD14/-159C/T* and *TLR4/+896A/G*. No epistatic effect was found in the case of atopic phenotype (data not shown).

Discussion

In this study, we found that the *CD14/-159C/T*, *TLR4/+896A/G*, and *TLR2/-16934A/T* polymorphisms did not affect either atopy or allergic disease susceptibility in a population of Polish children. To date, there have been no association studies investigating these polymorphisms within *TLR2* and *TLR4* genes in this population, and only 3 studies investigated *CD14* polymorphisms in relation to asthma.[35–37] To the best of our knowledge, this is also the first study in this population that attempts

Table 5. Association between CD14, TLR4 and TLR2 genotype combinations and asthma and allergic rhinitis

Genotype combinations	Control n [%]	Asthma			Allergic rhinitis		
		n [%]	p-value	OR (95% CI)	n [%]	p-value	OR (95% CI)
CD14 CC + TLR2 TT	2 (4.2)	1 (3.0)	1.000	1.00 (ref) –	1 (3.0)	0.565	1.00 (ref) –
CD14 CC + TLR2 TA/AA	11 (22.9)	9 (27.3)		2.08 (0.09÷46.2)	9 (27.3)		2.90 (0.23÷36.1)
CD14 CT/TT + TLR2 TT	9 (25.7)	13 (56.5)	0.037*	1.00 (ref) –	13 (56.5)	0.032*	1.00 (ref) –
CD14 CT/TT + TLR2 TA/AA	26 (74.3)	10 (43.5)		0.25* (0.07÷0.87)	10 (43.5)		0.31* (0.11÷0.88)
TLR4 AA + TLR2 TT	9 (18.8)	13 (39.4)	0.038*	1.00 (ref) –	13 (39.4)	0.030*	1.00 (ref) –
TLR4 AA + TLR2 TA/AA	35 (72.9)	15 (45.5)		0.27* (0.08÷0.85)	15 (45.5)		0.35* (0.13÷0.95)
TLR4 AG/GG + TLR2 TT	2 (50.0)	1 (20.0)	0.524	1.00 (ref) –	1 (20.0)	0.547	1.00 (ref) –
TLR4 AG/GG + TLR2 TA/AA	2 (50.0)	4 (80.0)		0.67 (0.03÷16.38)	4 (80.0)		1.83 (0.06÷54.9)

* statistically significant results; ref – reference; OR – odds ratio; CI – confidence interval.

to understand the gene–gene interactions based on polymorphisms. Among the large number of SNPs currently identified in the studied genes, we decided to cover SNPs that have proven and relevant functional consequences.[9–11] Although the correlations between polymorphisms in the *TLR2*, *TLR4* and *CD14* genes in allergic diseases have received considerable attention in recent years, the results are still contradictory.

The first hints that variations in genes which encode receptors of innate immunity may modulate the risk of allergy actually came from studies on *CD14*, but the results of the association studies remain controversial. Baldini et al.[17] reported an association of the *CD14-159/TT* genotype with higher sCD14 expression, lower total serum IgE and fewer mean positive SPTs in atopic patients. Numerous studies have replicated the results from the initial Tucson study, but no correlation between the *CD14/-159C/T* polymorphism and atopic diseases has been observed.[18–20,30] The lack of association between *CD14* genotypes and atopic diseases in our research is in line with the results of these studies. However, we could not find any association between *CD14* polymorphism and total serum IgE level or the number of positive SPTs. Similar results were obtained in a previous study on Polish children.[36] In addition, no such associations were found in either the Asian or Caucasian populations.[21,27,38–41] A significant correlation between the *CD14/-159C/T* polymorphism and asthma was not observed in another recent study among the Puerto Rican population, the population that exhibits the highest asthma prevalence of any racial or ethnic group.[42] On the other hand, other authors have reported a correlation of the *CD14-159/TT* genotype with a lower risk of developing asthma[17,31,35,43] or have even reported the opposite results.[32,33,44]

In the present study, the genotype and allele frequency of the *TLR4/+896A/G* polymorphism did not significantly differ between the allergic patients and the controls. Our results are consistent with studies in populations from Denmark,[30] Tunisia,[31] Egypt,[45] Korea,[46] and Japan[47] that did not find an effect of the *TLR4/+896A/G* polymorphism on the risk of asthma or atopy-related traits. In addition, similar results were obtained in both the Puerto Rican population[42] and a heterogeneous American cohort.[29] The lack of association between the *TLR4/+896A/G* polymorphism and asthma has also been confirmed by the latest meta-analysis.[23] In contrast, a previous meta-analysis of 8 studies showed a marginal association of *TLR4/+896A/G* with asthma risk, indicating that the major A allele might protect against asthma.[24] This is in agreement with a study among Swedish children suggesting that the AG genotype was associated with an increased risk of asthma, especially atopic asthma.[27] Yang et al.[28] observed a positive correlation of the *TLR4+896/G* allele with atopy severity in an asthmatic population, whereas another analysis of Turkish children showed that the heterozygosity of the *TLR4/+896A/G* polymorphism is associated with milder forms of asthma.[20]

When it comes to the *TLR2/-16934A/T* polymorphism, a large European study showed that farmers' children carrying a T allele in *TLR2/-16934* were less likely to have a diagnosis of asthma, atopic sensitization or hay fever symptoms.[26] In this study, we did not find any significant correlation between the *TLR2/-16934A/T* polymorphism and either atopy or the development of asthma/allergic rhinitis. This finding is in agreement with a study conducted on a population of young Danish farmers which did not demonstrate any effect of the *TLR2* polymorphisms on the risk of new-onset asthma and atopy.[30] Similarly, no association between the *TLR2/-16934A/T* polymorphism and asthma were found in the Puerto Rican population.[42] Interestingly, Kerkhof et al. showed in their PIAMA cohort study that children with at least 1 A allele in *TLR2/-16934*

had a significantly lower risk of doctor-diagnosed asthma.[48] A similar, but statistically insignificant trend was observed in our analysis for atopy as well as for asthma and allergic rhinitis.

One characteristic feature in the genetics of complex diseases is the low reproducibility and contradictory results of association studies. These conflicting results can be attributed to a lack of statistical power, differences in ethnicity and age, genetic heterogeneity, and environmental exposure. Multiple gene–gene interactions are also likely to contribute to such discrepancies. Gene–gene interactions seem to be biologically plausible, especially with regard to the genes which encode PRRs, form functional heterodimer networks, and take part in complex mechanisms of human innate and adaptive immunity. Reijmerink et al.[49] identified gene–gene interactions of TLR4-related pathway genes in connection with the development of asthma and atopy. Interestingly, significant gene–gene interactions were identified even with SNPs that did not demonstrate an effect on their own. Another study demonstrated that genes involved in the development and functioning of regulatory T cells, specifically IL2RA, TLR2, TGFBR2, and FOXP3, are associated with atopy and asthma when looking at gene–gene interaction.[50]

Therefore, we examined the possibility of a combined effect between the *CD14/-159C/T*, *TLR4/+896A/G* and *TLR2/-16934A/T* polymorphisms in relation to atopy-related traits. Interestingly, our results revealed that children heterozygous or homozygous for the *TLR2/-16934A* and *CD14/-159T* alleles had a significantly lower risk of asthma and allergic rhinitis than children carrying the same genotype for *CD14* who were homozygous for the *TLR2/-16934T* allele. Similar patterns were observed when looking at the combination of *TLR4* and *TLR2* polymorphisms: the co-occurring homozygous genotype for the wild-type *TLR4* allele and at least 1 *TLR2/-16934A* allele was associated with a reduced risk for asthma and allergic rhinitis. These findings suggest some possible epistatic effect of these polymorphisms on asthma and allergic rhinitis, because we could not find any significant correlations in the single-SNP analyses. Thus, it is possible that each variant has a small effect which is not strong enough by itself to cause such a complex disease as asthma or atopy. Our results provide support for our initial hypothesis that the combination of functional SNPs in more than one gene which encodes receptors of innate immunity may magnify the impact of disease or may indicate a new association.

A potential limitation of our study, typical for all case-control studies, is the relatively small sample size and, consequently, rather low statistical power, which may lead to false negative or fortuitous false positive results. An interaction analysis requires a large population; hence, our results should be interpreted with caution. However, the biological plausibility of the interactions between the genes for PRRs lends credibility to our results.

Conclusions

In summary, our study and the others mentioned above support the notion that the polymorphisms we tested may not play a crucial and decisive role in the development of atopic diseases by themselves, but our preliminary results suggest that their impact on the risk of asthma and allergic rhinitis might be modulated by gene–gene interactions. Further studies to confirm such interactions must be well-designed with adequate sample sizes in order to ensure enough statistical power. We conclude that some genetic associations might remain unnoticed when gene–gene interactions are not considered and we recommend that future studies examine combined genetic effects as well as primary ones.

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