

Original article

Rare TLR2 mutations reduce TLR2 receptor function and can increase atopy risk

Background: Common genetic variations in toll-like receptor 2 (TLR2), an innate pathogen recognition receptor, may influence the development of atopic diseases. So far, very little is known about the role of rare TLR2 mutations in these diseases.

Objective: We investigated the functional properties of six rare amino acid changes in TLR2 (and one amino acid change in a TLR2 pseudogene) and studied their effect on atopic sensitization and disease.

Methods: We identified rare TLR2 mutations leading to amino acid changes from databases. Functional effects of TLR2 variants were analyzed by NF- κ B-dependent luciferase reporter assay and interleukin-8 enzyme linked immunosorbent assay *in vitro*. The frequency of these mutations was determined in a random sample of the general population ($n = 368$). Association with atopic diseases were studied in a cross sectional German study population ($n = 3099$).

Results: Three out of six mutations in the TLR2 gene altered receptor activity *in vitro*. Out of these, only the minor allele of R753Q occurred reasonably frequent in the German population (minor allele frequency 3%). The risk to develop atopy increased by 50% in carriers of the 753Q allele ($P = 0.021$) and total ($P = 0.040$) as well as allergen specific serum IgE levels ($P = 0.011$) were significantly elevated.

Conclusion: The rare but functionally relevant mutation R753Q in TLR2 may significantly affect common conditions such as atopic sensitization in the general population.

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Introduction

Exposure to microbial stimuli seems to protect from the development of atopic sensitization and disease (1). This effect may be mediated to a substantial part by Toll-like receptors (TLRs), which mediate cellular recognition of microbial products and trigger innate and, as a consequence, adaptive immune responses upon activation (2). In a comprehensive analysis of common polymorphisms in the TLR system (human TLR1-10) we recently demonstrated that genetic variation in the TLR2 associated heterodimer network (consisting of TLR1, TLR6 and potentially TLR10) influences the occurrence of atopic asthma in a cross sectional population. However, in those populations we did not observe strong effects of common TLR2 variants on the development of atopic diseases (3).

In contrast, recent studies had suggested that a TLR2 promoter polymorphism could influence the risk to develop asthma under specific environmental conditions of high microbial exposure (4). At the same time, this

study indicated that an interplay between environmental exposures to TLR2 mediated stimuli and genetic variation in the TLR2 gene may determine susceptibility for asthma and atopy. Recently, it was also shown that a TLR2 agonist promotes Th1 response and ameliorates allergic airway response in mice (5).

Based on these observations it was hypothesized that a specific threshold change in TLR2 mediated signals may be necessary to develop atopic diseases. This threshold can be influenced by strong environmental exposures in combination with common but functionally weak genetic variants as shown by Eder et al. (4) but may also be modified by rare mutations leading to strong alterations of TLR2 associated pattern recognition, independent of exposure intensity to microbial or viral stimuli. Thus, we searched the innate immunity database (6) for mutations of the TLR2 gene putatively resulting in nonconservative changes in the respective amino acid sequence of the protein. The six rare mutations identified to lead to amino acid changes were tested for functionality *in vitro* using NF- κ B driven reporter gene assays and interleukin-8

(IL-8) enzyme-linked immunosorbent assay (ELISA). The frequency of these TLR2 mutations in the general population and their association with the development of atopic diseases in two independent populations of German children were determined ($n = 3099$).

Methods

Population description and phenotyping

Population description. Between 1995 and 1996, a cross-sectional study was performed in Germany as part of the International Study of Asthma and Allergies in Childhood (ISAAC phase II), to assess the prevalence of asthma and allergies in schoolchildren, age 9–11 years ($n = 5629$). All children of German origin with DNA available were included in this analysis ($n = 3099$). Informed written consent was obtained from parents and the study methods were approved by the ethics committees of Munich and Münster (study and data centers). The population and phenotyping methods have been described in detail before (7, 8).

Phenotyping. Self-administered questionnaires included the ISAAC core questions. Children whose parents reported a physician's diagnosis of asthma, or recurrent spastic or asthmatic bronchitis were classified as asthmatics. Children's classification of hay fever or atopic eczema was also based on parental report of a physician's diagnosis of the respective diseases. The sensitivity to six common aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Alternaria tenuis*, cat dander, mixed grass and tree pollen) was assessed by skin prick test (ALK, Horsholm, Denmark). A child was considered atopic if a wheal reaction of ≥ 3 mm occurred to one or more allergens after subtraction of the negative control. Total serum IgE levels were measured using the Imulite system (DPC Biermann, Bad Nauheim, Germany). Specific IgE against inhalative allergens (local grass pollen, birch pollen, mugwort pollen, *Dermatophagoides pteronyssinus*, cat dander, dog dander, *Cladosporium herbarum*) were measured in a range between 0.35–100 IU/ml (*SxI*; Phadia, Uppsala, Sweden). Current environmental smoke exposure was defined as any current environmental tobacco smoke exposure at the age of 9–11 years according to the information derived from parental questionnaires.

Laboratory methods

Genotyping. Genomic DNA was extracted from whole blood by a standard salting out method (9) and preamplified as described (10). DNA samples were genotyped using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, CA, USA). PCR assays and associated extension reactions were designed using the SPECTRODESIGNER software (Sequenom). All amplification and extension reaction conditions have been described previously (10) and specific primer sequences are given in Table 1. For quality control of genotyping procedures, DNA of all homozygous carriers of rare alleles were re-sequenced using an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA, USA) as tests for Hardy–Weinberg equilibrium are not sufficiently informative for rare mutations.

Mutagenesis. A wildtype (wt) human TLR2 expression plasmid with the original leader sequence replaced by a 5'-terminally fused trypsin leader followed by a Flag-tag coding sequence (pFLAG-CMV; Sigma-Aldrich, Munich, Germany) (11) was employed as template in overlap-PCR based mutagenesis (Stratagene, La Jolla, CA, USA) performed according to the supplier's protocol to introduce the specific TLR2 SNPs. Briefly, complementary primer pairs (30–40 bp), each of which carried the respective mutated/mismatch base in its center so that the 5' and 3' flanking sequences matched the wildtype TLR2 template plasmid insert sequence, were added to the template. The entire plasmid including the TLR2 coding sequence was amplified in 18 cycle PCR reactions using *Pfu* polymerase (11). All DNA constructs were subsequently sequenced.

Cell culture. The human embryonic kidney cell line (HEK) 293 was grown under regular mammalian cell culture conditions in Dulbecco's modified eagle medium (DMEM; PAA, Pasching, Austria) supplemented with 10% FCS (PAA) and standard antibiotics (PAA) (11).

Reporter gene assay. A total of 3×10^4 HEK293 cells were cultured on single wells of 96-well plates. HEK293 cells were cotransfected with (i) the cytomegalovirus (CMV)-promoter driven plasmid expressing our TLR2 variants; (ii) an NF- κ B recruiting endothelial-leukocyte adhesion molecule (ELAM)-1 promoter firefly luciferase construct; (iii) a renilla luciferase control reporter construct;

Table 1. Description of the investigated TLR2 protein changes and primers used for MALDI-TOF MS

TLR SNP	rs number	Alleles	PCR primer (first and second)	Extension primer	Minor allele frequency		
					<i>In silico</i> †	<i>EA</i> ‡	cross-sectional
T411I	rs5743699	C/T	ACG TTG GAT GTT GGA AAA AAC CGG AGA GAC ACG TTG GAT GCT GAC AAG TTT CAG GCA TAG	ATG TTA GTC AAG TTT TTC AGA	0.01	0	0.0003
R579H	rs5743703	G/A	ACG TTG GAT GTA CCT GTG TGA CTC TCC ATC ACG TTG GAT GAG TGC TGT CCT GTG ACA TTC	ATT CCG ACA CCG AGA GG	0.01	0	0
P631H	rs5743704	C/A	ACG TTG GAT GTC CAT GGC CTG TGG TAT ATG ACG TTG GAT GTA GCA GAT GTT CCT GCT GGG	CTC CTG CTG GGA GCT TTC CTG	0.03	0.04	0.04
R677W	rs5743706	A/T	Located in a TLR2 pseudogene and not further genotyped				n.a.
Y715 stop	rs5743707	T/A	ACG TTG GAT GCT TCA CTC AGG AGC AGC AAG ACG TTG GAT GAA AGA CGG AAA TGG GAG AAG	GAA ATG GGA GAA GTC CAG TTC	0.01	0	0
Y715N	rs5743705	T/G	ACG TTG GAT GAG ACG GAA ATG GGA GAA GTC ACG TTG GAT GGA CAC AAA ACT GTC TTT GTG C	ACC ACT CAC TCT TCA C	0.01	0	0.0005
R753Q	rs5743708	G/A	ACG TTG GAT GTT CTT CTG GAG CCC ATT GAG ACG TTG GAT GCC AGG TAG GTC TTG GTG TTC	TAG GTC TTG GTG TTC ATT ATC TTC	0.02	0.02	0.03

†Frequencies in samples of different ethnicities (Hispanic/African/European Americans).

‡European American samples ($n = 23$ each)

and (iv) a CD14 expression vector using the calcium phosphate precipitation method as previously described (11). After 24 h a lipopeptide, the prototypic TLR2 ligand tripalmitoylated hexapeptide (Pam₃CSK₄; EMC microcollections, Tuebingen, Germany) was added at successively increasing amount. After 16 h cells were lysed for measurements of reporter gene activity. Firefly luciferase activity was normalized by renilla activity values (11).

Enzyme linked immunosorbent assay. Before cells were lysed for reporter analysis (as described above), supernatants were collected and IL-8 concentrations were determined by ELISA (RnDsystems, Minneapolis, MN, USA) using colorimetry (TECAN, Crailsheim, Germany) according to supplier protocols. Concentrations below 1 pg/ml were below the detection limit.

Flow cytometry. A total of 1×10^5 HEK293 cells were transiently transfected with (i) DNA plasmids expressing TLR2 variants and the (ii) CD14 expression vector as previously described. Transfected HEK293 were incubated with Fc block (BD Pharmingen, Heidelberg, Germany) and stained with anti Flag mAb (M1, Sigma, Hamburg, Germany) or anti CD14 mAb (BD) and subjected to flow cytometry (CyAn ADP LX9; Dako, Glostrup, Denmark). Empty vector transfected cells were used as control.

Immunoblot analysis. A total of 1×10^5 HEK293, transiently transfected as described for flow cytometry, were lysed and lysate aliquots representing 5×10^5 cells were subjected to each lane of a 10% SDS-PAGE gel. The gel content was then blotted and blots were analyzed with polyclonal anti-Flag antiserum (Sigma-Aldrich, Munich, Germany) (11).

Statistical analyses and bioinformatics. Association between SNPs and dichotomous outcomes were evaluated using chi-square tests in a dominant model of the rare allele. Values for total serum IgE levels were first log-transformed and *t*-tests of log-transformed values were applied in the dominant model. Skin test measurements and specific serum IgE against a mix of aeroallergens (sx1) were dichotomized according to a predefined threshold (0.7 kU/l) and analyzed also using chi-square tests in a dominant model. Additionally, we calculated a model adjusted for age, sex and environmental tobacco smoke. For the adjusted models we give results of logistic or linear regression respectively. Significance of differences in supernatant IL-8 concentrations and NF- κ B-driven reporter gene activities were determined by the exact Wilcoxon-Mann-Whitney test. Mean values of two replicates per experiment were used and the analyses were stratified for the different stimulus conditions. After log-transformation of the raw values, normal distribution was obtained, thereby allowing multiple regression analysis. All tests were two-sided and the differences were considered significant when $P < 0.05$. Analyses were carried out with SAS (Version 9.1.3) and SAS/Genetics.

Results

Information on sequence variants in the TLR2 gene were retrieved from the publicly accessible data base of the innate immunity program of genetic application (<http://www.innateimmunity.net>). *In silico* analyses of TLR2 sequences identified seven infrequent amino acid changes within the TLR2 gene, one (R677W) of which was later discovered to reside within a TLR2 pseudogene (MAF 1-3%, Table 1).

To study the effects of mutations in the *TLR2* gene on TLR2 receptor function, equal expression levels of TLR2 variants and wildtype had to be ensured in the HEK293 reporter system first. Using immunoblot and

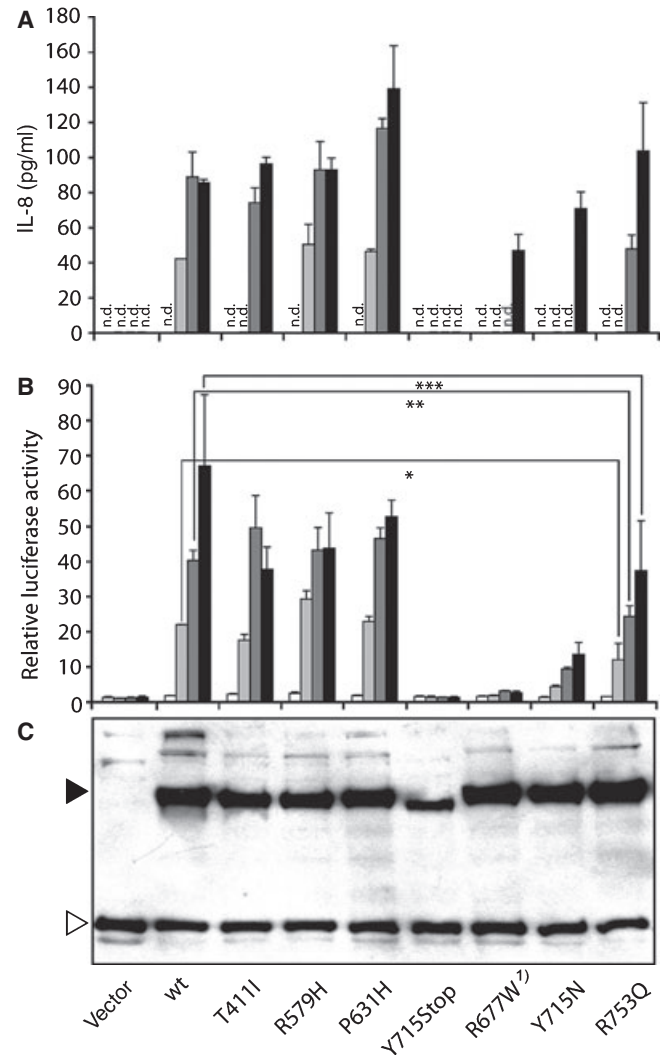


Figure 1. TLR2 variant expression levels, TLR2 signaling induced NF- κ B-driven reporter gene activity and IL-8 release. HEK293 cells transfected with TLR2 constructs expressed under CMV promoter control were challenged with a tri-palmitoylated hexapeptide (Pam₃CSK₄) at increasing concentrations (white columns, unstimulated; bright grey columns, 10 ng/ml; dark grey columns, 100 ng/ml; black columns, 1 µg/ml). After 16 h, (A) Expression of Flag-tagged TLR2 variants in transiently transfected HEK293 cells were analyzed by immunoblot (wt, wildtype TLR2; ►, Flag-specific protein band; unspecifically stained protein band; ▽, loading control). (B) NF- κ B-driven luciferase activity was determined by reporter gene assay and (C) IL-8 content in supernatants (n.d., not detectable) was analyzed by ELISA. Results illustrated are representative of equivalent results of at least six independent experiments, each performed in duplicates. Significance levels are displayed for the atopy associated variant R753Q only ($P < 0.1$: * $P = 0.011$, ** $P = 0.018$, *** $P = 0.073$). ¹located in a TLR2 pseudogene.

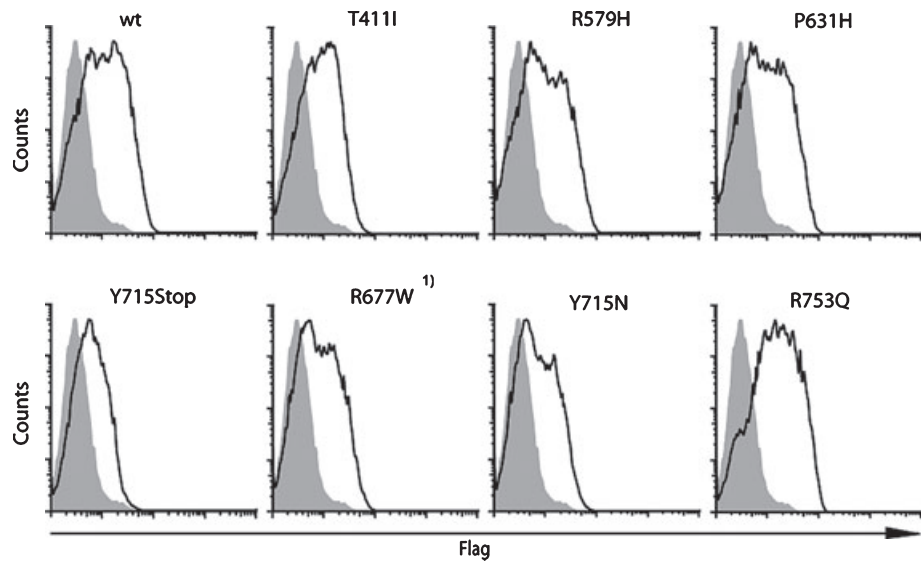


Figure 2. Analysis of TLR2 variant cell surface expression by flow cytometry. HEK293 cells transiently transfected with TLR2 constructs under CMV promoter control were stained in a flag tag specific manner using FITC labeled secondary mAb. Cell surface associated fluorescence intensities were analyzed (grey fields: empty vector transfected HEK293 cells; black lines: HEK293 cells expressing flag tagged TLR2 variants as indicated and untagged CD14). ¹⁾located in a TLR2 pseudogene.

flow cytometry analysis equal TLR2 variant expression levels were observed (Fig. 1c). Moreover, both CD14 and all N-terminally Flag-tagged wildtype and mutant TLR2 constructs were expressed to similar degrees at the cell surface as indicated by flow cytometry (Fig. 2 and data not shown).

By analyzing TLR2 variant mediated cell activation we found that TLR2 signaling activity was not altered by variants T411I, R579H, and P631H compared to wildtype TLR2 when assessed by NF-κB-driven reporter gene activation and IL-8 release assays. In contrast, Y715Stop, Y715N, R753Q and R677W (TLR2 pseudogene mutation) substantially reduced TLR2 activity in these assays

(Fig. 1). NF-κB-driven reporter gene activity was reduced by approximately 80% in the case of the pseudogene mutation R677W and 50% for R753Q throughout the range of different concentrations at which lipopeptide was applied (Fig. 1). Conclusively, luciferase activity was reduced by about 50% in cells that expressed the R753Q variant as compared to cells expressing wildtype TLR2 [geometric mean ratio: 0.45 (0.38–0.53), *P* < 0.001]. IL-8 release was diminished at low and intermediate lipopeptide levels. However, upon challenge with high dose lipopeptide, cells expressing either R753Q or Y715N released IL-8 to an extent that did not differ significantly from cells expressing wildtype TLR2.

Table 2. Dominant effects of TLR2 protein changes (rare variant) on total serum IgE levels and atopy

	Total IgE	Specific serum inhalative allergens (≥0.7)	Skin test reactivity (≥3 mm)	Asthma	Atopic eczema	Hay fever
	Geom. mean (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value	OR (95% CI) <i>P</i> -value
P631H	PP 69.2 (65.6–73.0) PH/HH 62.0 (52.3–73.5) 0.247	1036/3097 0.92 (0.69–1.21) 0.547	777/3002 0.85 (0.63–1.16) 0.318	272/3054 1.02 (0.65–1.62) 0.923	540/2994 0.88 (0.62–1.26) 0.485	280/3032 0.79 (0.48–1.31) 0.365
P631H adjusted*	0.220	0.91 (0.69–1.21) 0.535	0.83 (0.61–1.15) 0.262	0.99 (0.62–1.58) 0.954	0.90 (0.63–1.29) 0.5804	0.77 (0.46–1.28) 0.310
R753Q	RR 68.8 (65.3–72.6) RQ/QQ 87.9 (70.4–109.8) 0.040	1.54 (1.10–2.14) 0.011	1.52 (1.06–2.17) 0.021	0.67 (0.33–1.32) 0.241	1.16 (0.77–1.76) 0.473	1.37 (0.81–2.31) 0.239
R753Q adjusted*	0.043	1.57 (1.12–2.20) 0.009	1.53 (1.06–2.19) 0.023	0.68 (0.34–1.36) 0.272	1.16 (0.76–1.77) 0.488	1.41 (0.83–2.39) 0.200

95% CI, 95% confidence interval, OR, odds ratio.
*Adjusted for sex, age and environmental tobacco smoke.

Subsequently, we evaluated the presence of the six TLR2 mutations in German individuals ($n = 368$) randomly selected from the cross sectional ISAAC population. Four of these six mutations were initially described only in African and Hispanic American descent, but not in Europeans (Table 1). R579H and Y715Stop were not at all present in our German subpopulation of 368 children and not followed further. T411I and Y715N were observed at a minor allele frequency of only 0.0003 and 0.0005 in our total study population of 3099 children. P631H and R753Q, the two non synonymous amino acid changes originally described in Americans of European descent, were also present in our population based sample at a reasonable frequency (Table 1). P631H and R753Q showed a very low LD ($R^2 = 0.001$).

In a next step, we studied associations of the P631H and R753Q mutations with atopic phenotypes. 753Q significantly increased the risk for atopy as measured by skin prick test in the cross sectional population ($n = 3099$) by approximately 50% (Table 2). Additionally, carriers of this protein variant showed elevated total serum IgE levels and more allergen specific serum IgE (Table 2). In contrast, 631H had no significant effect on any of the investigated atopic phenotypes in our population. The results remained significant for R753Q when we adjusted our models for sex, age and environmental tobacco smoke. This was also true for the linear model (data not shown).

Discussion

Here we investigated the role of rare TLR2 amino acid changes in TLR2 receptor function, their prevalence in the general German population and their influence on the development of atopic sensitization and disease. Three of the investigated mutations did not change TLR2 activity. TLR2 activity in our *in vitro* test system was markedly reduced by Y715Stop, R677W, Y715N, and R753Q in TLR2 and the TLR2 pseudogene mutation R677W (when cloned into a TLR2 plasmid). Only four TLR2 mutations could be identified in a cross sectional sample of the German population and only P631H and R753Q were found at reasonable frequencies, indicating that all other putative TLR2 mutations are very infrequent in the general population. R753Q was associated with diminished TLR2 activity in the *in vitro* experiments and also influenced the risk to develop atopic sensitization. As expected, P631H, which had not influenced TLR2 activity *in vitro*, was not associated with atopic outcomes.

This study was based on the assumption that in complex diseases with multiple genetic factors involved, rare genetic changes in key genes with strong functional implication might also exert significant effects on disease detectable at the population level. Thus, we selected TLR2 as a potential gene of interest, as it represents a hub in the TLR system, capable of forming heterodimers

with other TLRs such as TLR1, TLR6 and possibly TLR10 (2). Next, mutations leading to nonsynonymous amino acid changes in the TLR2 gene were identified, as mutations changing the structure of the TLR2 protein have a high probability to significantly alter the function of the receptor. TLR2 mutations were selected using data available from an extensive and systematic mutation screening program of innate immunity genes. As this program was based on the screening of not more than 25 individuals, it might be argued that very rare mutations with minor allele frequencies below 1% could have been missed by this approach. However, even our relatively large population sample available for genotyping would have been too small for association studies between mutations with minor allele frequencies of less than 1% and common conditions such as atopic sensitization and disease.

Functional effects of these TLR2 mutations were studied in a well established TLR2 *in vitro* assay which had been used previously to determine TLR2 activity under a variety of conditions (11). In these *in vitro* experiments the Y715Stop variant was completely inactive (Fig. 1). This was expectable since it is known that deletion of the C-terminal FW-motif abrogates NF- κ B activation through TLR2 (12). For all other constructs, relative luciferase activities as well as IL-8 release both resulting from TLR2 variant mediated NF- κ B activation were analyzable in a comparable manner.

R677W, initially thought to be located in TLR2 and only later found to reside in a TLR2 pseudogene (13) was originally associated with increased leprosy incidence (14, 15). This mutant, when expressed in TLR2, showed a signaling reduction nearly as strong as Y715Stop when compared to wildtype TLR2 (Fig. 1). However, as this SNP is not located in TLR2 *in vivo*, it was not followed up in our association studies.

753Q resulted in reduced TLR2 activity. The 753Q mutant displayed both diminished NF- κ B activity and IL-8 release upon challenge with the synthetic bacterial lipopeptide analogue Pam₃CSK₄ (Fig. 1) while being expressed at the cell membrane at comparable levels as the wildtype construct (Fig. 2). Thus, we hypothesized that these mutations reducing the activity of the TLR2 receptor may also contribute to a decreased Th1 immune response and thus permit more pronounced Th2 responses, leading to the development of atopy and atopic diseases.

To test if these TLR2 variants could actually affect the risk for atopic sensitization and disease, it was necessary to establish if these mutations are present in our study population at detectable frequencies. Therefore, all TLR2 mutations were genotyped in a random sub-sample of 368 individuals. Only mutations found at least once in this sub-sample were genotyped in the complete study population. Only four of the investigated mutations (T411I, P631H, Y715N and R753Q) were present in the random sample at allele frequencies comparable to recent studies

of rare TLR2 mutations in another population of European descent (16, 17). Two of these four mutations, 715N and 753Q, were also associated with reduced TLR2 activity in our *in vitro* test system.

TLR2 variant 753Q with a minor allele frequency of 3%, significantly and consistently increased the risk for elevated total serum IgE levels and atopic sensitization determined by skin prick test and RAST measurements in our study population. Previous reports indicated association of 753Q with staphylococcal infections (18) and atopic eczema (11.5% of patients with atopic eczema were identified as heterozygous carriers) (19). This patient subgroup had a 55% risk for cutaneous *S. aureus* colonization and was defined by increased disease severity and highest IgE levels. However, a more recent study could not confirm an association between 753Q and atopic eczema (17) and neither could we, which may be attributable to power limitations in assessing rare effects on atopic diseases which are less frequent than atopic sensitization in our population. Thus, a role of R753Q in atopic diseases can neither be excluded nor proven.

Surprisingly, the effect of the mutation 753Q on TLR2 receptor signaling is strong enough to affect the risk for atopy in our general population sample. In contrast, associations between common TLR2 SNPs with putatively less significant functional effects and atopic diseases have been reported to be dependent on microbial exposure (4).

The fact that infrequent mutations may be associated with common diseases needs special consideration, as a new generation of genome wide association studies is

emerging (20), predominantly based on frequent haplotype tagging polymorphisms. Some important but minor genetic effects based on rare mutations would be missed if genome wide association studies approaches would be used exclusively to study asthma and allergy genetics.

Acknowledgments

Michael Kormann participated in genotyping (together with Norman Klopp and Thomas Illig), interpreted data and drafted the first version of the manuscript; Ruth Ferstl, Stephan Spiller, and Carsten Kirschning characterized TLR2 variants *in vitro*; Martin Depner performed statistical analyses; Christian Vogelberg was involved in data collection; Erika von Mutius contributed to the collection of data and data interpretation; Michael Kabesch developed the study design, contributed to data collection, supervised genotyping, interpreted data and wrote the final version of the manuscript. We thank Sonja Zeilinger and Sylvia Fichte for technical assistance.

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All authors declare that they have no competing financial or personal interests.

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