

TLR2 AND TLR4 GENE POLYMORPHISMS AND ATOPIC DERMATITIS IN ITALIAN CHILDREN: A MULTICENTER STUDY

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Background Genetic factors have an important role in atopic dermatitis (AD) predisposition. Toll like receptor (TLR) are important mediators between environment and immune system. There are inconsonant studies about TLR polymorphisms in AD. **Objective** This study examined whether single nucleotide polymorphisms (SNPs) in the genes for TLR2 and TLR4 could be associated with the AD phenotypes and with its clinical severity in a large group of Italian children. **Methods** 187 children with AD and 150 healthy children were recruited. AD severity was assessed by SCORAD. TLR2 (A-16934T and R753Q polymorphisms) and TLR4 (D299G and T399I SNPs) were genotyped by PCR-RFLP. **Results** The frequency of the R753Q was significantly higher in AD children (16.0%) compared with controls (6.0%, $P=0.004$; OR 2.99, 95%CI 1.39-6.41; RR 1.46, 95%CI 1.14-1.69). AD patients a significantly different frequency of the D299G SNP (14.9%) in comparison with the controls (6.6%, $P=0.01$; OR 2.46, 95%CI 1.17-5.17; RR 2.24, 95%CI 1.15-4.45). **Conclusion** Children with AD may have a distinct genotype and the TLR-2 R753Q SNP was prevalent in a subset of patients with AD characterized by a more severe clinical picture.

Atopic dermatitis (AD) is an inflammatory skin disease characterized by pruritus and chronic or relapsing eczematous lesions that commonly presents during early infancy and affect up to 16% of children (1). AD is thought to represent an interaction between both genetic and environmental influences with trigger factors, including irritants, allergens and microorganisms. There is substantial evidence to suppose that genetic factors have an important role in AD predisposition (2). The risk of having atopic disease (such as AD, asthma, allergic rhinitis, etc.) for a child is 50% if one parent is allergic, rising to 75% if both parents are allergic. In addition, the concordance in monozygotic twins is 72%, whereas it is 23% in dizygotic twins (3). In industrialized countries, AD prevalence has increased during the past decades (4), and it has been postulated by the so-called "hygiene hypothesis" that the lack of exposure to microbial products in early infancy might, at least in part, be responsible for

this increase (5). Prospective studies supported an inverse relationship between AD occurrence and exposure to endotoxin, a cell membrane component of Gram negative bacteria, early day-care attendance, and pet ownership (6).

Recognition of microbial products, such as endotoxin, is mediated by the innate immune system in which Toll-like receptors (TLRs) are directly involved. TLRs are known to function as mediators between innate and adaptive immunity via induction of dendritic cell (DC) maturation and activation of naïve T cells primarily skewing the adaptive immune response towards Th1 polarization (7). Indeed, TLRs recognize various pathogen-associated molecular patterns (PAMPs) of microorganisms, such as lipopeptide, lipopolysaccharide (LPS), RNA and methylated CpG DNA (8), leading to an inflammatory response by inducing a cascade of interleukins and other pro-inflammatory mediators (9).

Key words: Atopic dermatitis, Toll like receptor 2 gene, Toll like receptor 4 gene, polymorphism

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0394-6320 (2011)

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In the skin, keratinocytes are the first responders to external invaders and serve as initiators in innate immunity to produce cytokines, chemokines, and antimicrobial peptides (10-12). Keratinocytes have been shown to express TLRs 1, 6 and 9, which can help keratinocytes to act as first-responder against pathogenic microorganisms (13-15). However, the induction and detection of TLRs remain unclear or anyway controversial among the previous reports.

It has been found a constant expression of TLR2 on the surface of keratinocytes (16). TLR2, in association with TLR1 and 6, interacts with a series of cell wall components deriving from Gram-positive bacteria such as lipopeptides and peptidoglycan (PGN) (7,17). PGN is a major constituent of the cell wall of Gram-positive bacteria which induces signal transduction via TLR2, NOD1 (Card4) and NOD2 (Card15) receptors, respectively. NOD molecules are a family of intracellular pattern recognition proteins involved in bacterial detection (18,19).

TLR4 functions as the main receptor for LPS of Gram-negative bacteria (20). Song et al (21) found that human keratinocytes express functional CD14 and TLR4. The binding of the lipopolysaccharide with CD14 and TLR4 expressed on keratinocytes resulted in a rapid intracellular Ca^{2+} -dependent response, nuclear factor- κB nuclear translocation, and secretion of pro-inflammatory cytokines and chemokines. However, the expression of TLR4 and the response to LPS have been controversially reported (16,21).

In previous studies, associations of the TLR2 polymorphisms -16934A>T with asthma (22) and R753Q polymorphisms with severe AD (23), as well as the TLR4 polymorphisms D299G with asthma (24) and T399I ones with a modified response to endotoxin (25) have been reported.

Therefore, in a candidate gene approach, the aim of this study was to examine whether single nucleotide polymorphisms (SNPs) in the genes for TLR2 and TLR4 could be associated with the AD phenotypes and with its clinical severity in a large group of Italian children.

PATIENTS AND METHODS

The study was carried out from 2010 to 2011. Globally, 187 Italian children (96 boys and 91 girls; median age 8.0 years, range 0.5–12) with AD were selected and enrolled (table 1). They were recruited among children attending the Departments of Paediatrics of 4 Italian University Hospitals: Catania, Messina, Napoli and Pavia. A written informed consent was obtained from the children's parents.

Inclusion criteria were the presence of AD, diagnosed according to validated criteria (26), in active status as

defined by SCORing Atopic Dermatitis (SCORAD) (27). In our study we selected patients suffering from AD alone, without other types of allergies.

Atopy was defined by the presence of these following criteria: (i) a positive skin prick test (weal diameter ≥ 3 mm) to one or more food and/or inhalant allergens; and (ii) raised circulating total IgE (cut-off > 100 IU/ml).

Skin prick test was performed as stated by the European Academy of Allergy and Clinical Immunology (28) and carried out with a standard inhalant allergen panel (dust mites, Graminaceae, Parietaria, *Europea olea*, *Alternaria*, cat epithelium) and against food allergens (milk, egg, fish, wheat, peanuts, tomato and peach) (Soluprick ALK, Horsholm, Denmark).

Total and allergen-specific serum IgE concentrations were measured using a quantitative assay (CAP System; Phadia, Uppsala, Sweden). The severity of AD was assessed using the SCORAD Index (27) for the evaluation of extensiveness of skin disease: head and neck, each arm, front and back of each leg, the four trunk quadrants and the genitalia. Objective signs (erythema, oedema/papulation, oozing/crusts, excoriation, lichenification, and xerosis) and subjective symptoms (pruritus and sleep loss) were recorded. AD was considered as mild if the SCORAD Index was < 25 , moderate if 25–50, and severe if > 50 (27).

Each child had a steroid-free interval of at least 4 weeks, and no patient received systemic or topical antibiotic treatment before the examination. Furthermore, none of the patients had acute bacterial superinfection.

All patients were clinically observed every 4 months for 1 year by the same supervisor for each center, who was trained in SCORAD Index evaluation and unaware of the aims of the study.

The controls were 150 non-allergic unrelated outpatients (77 boys and 73 girls; median age 9.0 years, range 1–13) with no history of allergic diseases, such as asthma, AD or allergic rhinitis (table 1). They had to have negative responses to inhalant and food allergens on skin prick tests and serum test. Exclusion criteria for the control group were a positive family or individual history of AD, allergic rhinoconjunctivitis and/or allergic asthma, and serum total IgE levels > 3.5 kIU/l.

Genotype assessment

DNA extraction

Genomic DNA was isolated from 1 ml of peripheral blood anticoagulated with EDTA as previously described (29)

Genotyping of the TLR2 polymorphisms

To detect the A-16934T and R753Q polymorphisms, a polymerase chain reaction (PCR) was performed using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life

Sciences Division, UK), 400 $\mu\text{mol/L}$ dNTPs, and 0.1 $\mu\text{mol/L}$ of each primer in a total volume of 25 μL . The specific primers PCR and the restriction enzymes for each SNP are given in Table 2.

For A-16934T polymorphism, after an initial denaturation for 5 min at 94°C, PCR was performed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 58°C for 40 sec, primer extension at 72°C for 30 sec. The final extension was performed at 72°C for 7 min. PCR reactions were carried out using a GeneAmp PCR system 2700 (Applied Biosystem, CA, USA). Genotyping of this SNP was performed by enzymatic digestion with Hpy188 III (New England Biolabs, Ipswich, MA, USA) at 37°C, overnight. After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

For R753Q polymorphism, the reaction for the common allele (G), with TLR2-F, TLR2-R and TLR-G, and then the reaction for the rare allele (A), with TLR2-F, TLR2-R and TLR2-A were performed in a final volume of 50 microliter under the following conditions: 4 min at 94°C followed by 30 cycles of denaturing at 94°C for 45 sec, annealing at 62°C for 60 sec, primer extension at 72°C for 45 sec. The final extension was performed at 72°C for 7 min. PCR reactions were carried out using a GeneAmp PCR system 2700 (Applied Biosystem, CA, USA).

PCR product were subjected to electrophoresis in a 2% agarose gel and stained with ethidium bromide. The 470-bp PCR product was amplified using TLR2-F and TLR2-R, and the 328 bp product using TLR2-A and TLR2-F, or TLR2-G and TLR2-F.

Wild-type/mutant genotype was confirmed by automatic sequencing using the ABI-PRISM Big Dye™ Terminator v. 3.0 Cycle sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). The sequencing products were purified using DyeEx Spin Kits (Qiagen) and visualized on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA).

Genotyping of the TLR4 polymorphisms

The two D299G and T399I SNPs of the TLR4 gene were determined by PCR-RFLP. The specific primers PCR and the restriction enzymes for each SNP are given in Table 2.

PCR was performed using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 $\mu\text{mol/L}$ dNTPs, and 0.1 $\mu\text{mol/L}$ of each primer in a total volume of 25 μL .

For D299G SNP, cycle conditions were an initial denaturation for 5 min at 95°C, followed by 32 cycles of denaturing at 95°C for 30 s, annealing at 51°C for 30 s, primer extension at 72°C for 30 s, followed by a

final extension at 72°C for 7 min. For T399I SNP, cycle conditions were an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturing at 95°C for 45 s, annealing at 55°C for 30 s, primer extension at 72°C for 45 s, followed by a final extension at 72°C for 7 min.

The amplified samples of TLR4 gene D299G and T399I SNPs were digested at 37°C, overnight, with the NcoI and HinfI restriction enzymes (New England Biolabs, Ipswich, MA, USA), respectively.

After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA).

As previously described here, the results of enzymatic digestion were confirmed by DNA sequence analysis of representative samples of each SNP (ABI Prism 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Data are given as mean values $\pm$ SD. Allele and genotypes frequencies in patients and in controls were compared by χ^2 test or Fisher exact test, when an expected value was <0.5 ; P values were considered significant at a level of <0.05 . Odds ratio (OR), RR (Relative Risk) and P values were calculated using a standard package STATA 9 (30).

Allele frequencies were tested for the Hardy-Weinberg equilibrium (30). Cases and controls were compared using Pearson's χ^2 test.

RESULTS

Allele frequencies in AD children

TLR2 gene SNPs

In AD patients, the frequency of the homozygous A/A genotype was significantly increased (36.8%) than in controls (24.0%) ($P=0.03$) (Table 3; Figure 1).

The frequency of the R753Q was significantly higher in AD children (16.0%) compared with controls (6.0%, $P=0.004$; OR 2.99, 95%CI 1.39-6.41; RR 1.46, 95%CI 1.14-1.69). No homozygous carriers of this TLR2 SNP were found in the study and control populations (Table 3; Fig. 2).

The TLR2 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in control subjects.

TLR4 gene SNPs

The results of the genotype analyses in 187 AD children and in 150 control subjects with regard to the TLR4 D299G and T399I SNPs are shown in Table 4.

AD patients a significantly different frequency of the D299G SNP (14.9%) in comparison with the controls (6.6%, $P=0.01$; OR 2.46, 95%CI 1.17-5.17; RR 2.24;

95%CI 1.15-4.45) (Fig.3).

The T399I SNP allele frequency was not significantly different between AD children (7.5%) and control group (7.3%, $P > 0.05$; OR 1.02; 95%CI 0.45-2.28; RR 1.01, 95%CI 0.66-1.34). No homozygous carriers of the two SNPs were found in the study and control populations (Fig.4).

The TLR4 allele frequencies were in Hardy-Weinberg equilibrium in all patients and in the control group.

Genotype-phenotype correlations in two subgroups of AD children with mild to moderate and severe atopy.

When the contribution of each SNP of the TLR2 gene was investigated, the major support to the genotype-phenotype correlation could be ascribed to the R753Q allele. In particular, in two subgroups of AD children with mild to moderate and severe AD phenotypes, the occurrence of one risk allele of R753Q was associated with severe AD phenotype ($P = 0.002$; OR 3.45; 95%CI 1.56-7.63; RR 2.14; 95%CI 1.35-3.10). The TLR2 A-16934 allele was not found to be associated with the severity of AD, as well as TLR4 D299G and T399I SNPs.

DISCUSSION

This study investigated the prevalence of TLR2 and TLR4 genetic variants in a large group of Italian children affected by atopic dermatitis and resident in different Italian regions. Moreover, it has been evaluated the eventual association of TLR2 and TLR4 SNPs with severity/phenotype of the disease.

TLR2 and TLR4 SNPs were extensively studied in the context of atopy and allergic disorders, including AD, but the data are very conflicting (22,23, 31-36).

To our knowledge, this is the first analysis of TLR2 and TLR4 SNPs in Italian AD children. This study identified the common TLR2 -16934A>T variant as the most frequent in children affected by AD, using a candidate gene approach in a well-characterized cohort of 187 AD children and 150 healthy controls. This study also demonstrated that the TLR2 R753Q SNP was more prevalent in AD children than in control subjects. However, the major contribute to AD was made by TLR2 R753Q SNP.

The TLR2 -16934 SNP is located near to an evolutionary conserved sequence and to several transcription factor binding sites, including retinoid x-receptors (RXRs), AP4, NF- κ B, and Interferon regulating factor (31). Interestingly, for the 'A' allele in position TLR2 gene -16934, no additional binding sites for AP4 and neuro 'D' were predicted. The presence of several transcription factor binding sites in an evolutionary

conserved sequence indicates that this region is transcriptionally relevant, and potentially genetically or transcriptionally imprinted.

So far, little efforts were made to examine the functional consequences of the TLR2 -16934A>T and R753Q mutations. Eder et al (22) reported an association between TLR2 gene -16934 A>T SNP and asthma, current asthma symptoms, atopy or current hay fever symptoms in farmers' children. The 'T' allele in the promoter region of the TLR2 gene was proposed protective of these diseases. No such association was found in non farmers'. A recent study reported an increased representation of the A-allele in position -16934 of the TLR2 gene among German AD adult patients with severe AD genotype (SCOAD>50) when compared with those with mild-to-moderate (SCORAD $\leq$ 50) (31). Weidinger et al (35) examined a large cohort of 275 German white parent-offspring trios affected by asthma, atopic eczema, or extrinsic atopic eczema. Their data showed that TLR2 and TLR4 gene SNPs do not confer atopic eczema susceptibility to the patient group.

To our knowledge, this is the first multicenter study performed in a group of AD Italian children

In our patients, we found an increased frequency of the homozygous A/A genotype than in controls. However, no significant difference was found between the two subgroups of mild-to-moderate AD and severe AD phenotype. The discrepancies between the results obtained in adults (oh et al) and those obtained in parent-offspring trios, and currently by us, might result from different study designs.

Our data suggest that TLR2 gene -16934 A allele could be a genetic factor of susceptibility to AD. The immediate molecular mechanism underlying the clinical observation remain unknown. Carriers of the -16934T allele were speculated to have increased expression of TLR2 on the surface of antigen-presenting cells because of putative regulatory molecular effect of -16934A>T polymorphism, resulting in a reduced risk of asthma or allergic disease (22). Indeed, Potaczek et al (32) found that reporter constructs carrying the -16934T allele shown higher transcriptionally activity than those having the -16934A allele. Probably, the T allele binds nuclear proteins corresponding to some unknown activating transcription factor. It could to hypothesize that other TLR2 SNPs in linkage disequilibrium with the -16934T allele may contribute to development of AD.

Moreover, a high frequency (12%) of adult AD, whose skin is known to be colonized with *S. aureus* who carried the TLR2 R753Q SNP, was found (35). The TLR2 R753Q mutation was also associated with more severe phenotype than in AD patients without this mutation (23). It has been shown that TLR2 expression was most severely impaired

Table 1. Demographic characteristics of Italian atopic dermatitis (AD) patients and AD negative healthy control subjects

	<u>AD</u>	<u>AD negative</u>
	Number (%)	
Total	187	150
Gender		
Male	96 (51.3)	77 (51.3)
Female	91 (48.7)	73 (48.7)
Age*	8.0 (0.5-12)	9.0 (1.0-13)
SCORAD°	34(+/-18)	n.d.
Total IgE (kU/l)	782((+/-30)	≤3.5 n.a.
AD stage		
Mild to moderate	132 (70,6%)	
Severe	55 (29,4%)	

*median age in years

°mean (+/- SEM, standard error of the mean)

Table 2 Primers sequences and restriction enzymes used for genotyping TLR2 and TLR4 genes

Gene locus	SNPs		Sequence	Restriction Enzyme
TLR2	-16934A>T	F o r	TLR2-F 5' AACAGAAATTTATCCATTCATGGTT 3'	Hpy188 III
		Rev	TLR2-R 5' AGCAGTTTATTGTGAGAATGAGTTT 3'	
	R753Q	For	TLR2-F 5' TATGGTCCAGGAGCTGGAGA 3'	
		Rev	TLR2-R 5' TGACATAAAGATCCCAACTAGACAA 3'	
		For	TLR2-G 5' GGTCTTGGTGTTCATTATCTTCC 3'	
		Rev	TLR2-A 5' GGTCTTGGTGTTCATTATCTTCT 3'	
TLR4	D299G	For	TLR4-F 5' GATTAGCATACTTAGACTACTACCTCCATG3'	NcoI
	T399I	Rev	TLR4-R 5' GTTAACTAATTCTAAATGTTGCCATCC 3'	HinfI
		For	TLR4-F 5' GGTGCTGTTCTCAAAGTGATTTGGGAGAA 3'	
		Rev	TLR4-R 5' CCTGAAGACTGGAGAGTGAGTTAAATGCT 3'	

in patients carrying the R753Q missense mutation. In this study, the TLR2 R753Q SNP was significantly associated to severe phenotype of AD. Our data are similar to that reported by other studies (23), but are in contrast with a previous large family-based study (35). However, because there is a substantial variation in frequencies of both genetic and non-genetic risk factors among

different populations, the findings of this study cannot be generalized to other populations.

Therefore, one may hypothesize that arginine in position 753 might support TLR2 delivery to cell surface (38).

For TLR4, two clinically relevant polymorphisms, D299G and T399I, have been described (39).

Table 3 Genotype and allele frequencies of TLR2 -16934A>T and R753Q SNPs in 187 Italian AD children and controls.

SNP	AD (n=187)	allele frequency (%)	controls (n.150)	allele frequency (%)	P
TLR2					
-16934A>T					
A/A	69 (36.8%)	18.4	36 (24.0%)	12.0	0.03
A/T	80 (42.7%)		75 (50.0%)		
T/T	38 (20.5%)		39 (26.0%)		
R753Q					
R/R	157 (84.0%)	8.0	141 (91.2%)	3.0	0.004
R/Q	30 (16.0%)		9 (6.0%)		
Q/Q	0		0		

No patients homozygous for TLR2 R753Q SNPs were found in this study population. P, AD patients vs control group.

Table 4 Genotype and allele frequencies of TLR4 D299G and T399I SNPs in 187 Italian AD children and controls

SNP	AD (n=187)	allele frequency (%)	controls (n.150)	allele frequency (%)	P
TLR4					
D299G					
Wild-type	159 (85.1%)	7.5	140 (93.4%)	3.3%	0.01
Heterozygous	28 (14.9%)		10 (6.6%)		
Mutant homozygous	0		0		
T399I					
Wild-type	173 (92.5%)	3.7	139 (92.7%)	3.6	n.s.
Heterozygous	14 (7.5%)		11 (7.3%)		
Mutant homozygous	0		0		

No patients homozygous for TLR4 D299G and T399I SNPs were found in this study population. P, AD patients vs control group.

They were located within the extracellular domain and were found in patients with septic shock, particularly when such shock was due to gram-negative infection (40). Furthermore, these common co-segregating TLR4 RFLPs were linked to LPS hypo-responsiveness (41).

In the present AD children, the TLR4 D299G RFLP had a prevalence of 14.9%, whereas in the control group the genotype frequency of this RFLP was 6.6%. Therefore, the TLR4 D299G polymorphism was significantly associated to AD in these children. In contrast, in patients with AD the genotype frequency of the TLR4 T399I SNP did not differ from the control subjects.

Both TLR4 SNPs were not associated with the different score of the AD severity. Similar results have been shown by Ahmad-Nejad et al (23).

This study complements and extends the knowledge on the association of TLR2 and TLR4 SNPs with AD. Even though several case/control studies have examined TLR2 and TLR4 SNPs in other atopic diseases like allergic asthma or rhinoconjunctivitis, data on their association with AD is still scarce. In particular, future studies will be directed at further analyzing the molecular effects of TLR2 SNPs and assessing whether these SNPs might be a useful predictive markers for a severe phenotype in at least a subgroup of patients with AD. In addition, this study provided an interesting finding that may be relevant from a clinical point of view: the occurrence of one risk allele of R753Q was associated with severe AD phenotype (OR 3.45 and RR 2.14).

In conclusion this study provided evidence that children with AD may have a distinct genotype and the TLR-2 R753Q SNP was prevalent in a subset of patients with AD characterized by a more severe clinical picture. Analysis of the effect of this variation on disease severity and potential gene/environment interactions might be a promising approach since gene/environment interactions have been described for other TLR genes.

REFERENCES

- Williams H, Robertson C, Stewart A, Ait-Khaled N, Anabwani G, Anderson R, et al. Worldwide variations in the prevalence of symptoms of atopic eczema in the International Study of Asthma and Allergies in Childhood. *J Allergy Clin Immunol* 1999; 103(1 Pt 1):125-38.
- Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev* 2011; 242(1):233-46.
- Larsen FS, Holm NV, Henningsen K. Atopic dermatitis. A genetic-epidemiologic study in a population-based twin sample. *J Am Acad Dermatol* 1986;15(3):487-94.
- Williams HC. Epidemiology of atopic dermatitis. *Clin Exp Dermatol* 2000;25(7):522-9.
- Borchers AT, Keen CL, Gershwin ME. Hope for the hygiene hypothesis: when the dirt hits the fan. *J Asthma* 2005;42(4):225-47.
- Kabesch M, Lauener RP. Why Old McDonald had a farm but no allergies: genes, environments, and the hygiene hypothesis. *J Leukoc Biol*. 2004;75(3):383-7.
- Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* 2002;20:197-216.
- Akira S. Toll-like receptor signaling. *J Biol Chem* 2003;278(40):38105-8.
- Takeda K, Akira S. TLR signaling pathways. *Semin Immunol* 2004;16(1):3-9.
- Dorschner RA, Pestonjamas VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, et al. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. *J Invest Dermatol* 2001;117(1):91-7.
- Ali RS, Falconer A, Ikram M, Bissett CE, Cerio R, Quinn AG. Expression of the peptide antibiotics human beta defensin-1 and human beta defensin-2 in normal human skin. *J Invest Dermatol* 2001;117(1):106-11.
- Sugita K, Kabashima K, Atarashi K, Shimauchi T, Kobayashi M, Tokura Y. Innate immunity mediated by epidermal keratinocytes promotes acquired immunity involving Langerhans cells and T cells in the skin. *Clin Exp Immunol* 2007;147(1):176-83.
- Köllisch G, Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J, et al. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 2005;114(4):531-41.
- Begon E, Michel L, Flageul B, Beaudoin I, Jean-Louis F, Bachelez H, et al. Expression, subcellular localization and cytokine modulation of Toll-like receptors (TLRs) in normal human keratinocytes: TLR2 up-regulation in psoriatic skin. *Eur J Dermatol*;17(6):497-506.
- Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitemaker JH, Kapsenberg ML, et al. Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol* 2007;127(2):331-41.
- Kawai K, Shimura H, Minagawa M, Ito A, Tomiyama K, Ito M. Expression of functional Toll-like receptor 2 on human epidermal keratinocytes. *J Dermatol Sci* 2002;30(3):185-94.
- Schumann RR. Host cell-pathogen interface: molecular mechanisms and genetics. *Vaccine* 2004;22 Suppl 1:S21-4.
- Girardin SE, Travassos LH, Hervé M, Blanot D, Boneca IG, Philpott DJ, et al. Peptidoglycan molecular

- requirements allowing detection by Nod1 and Nod2. *J Biol Chem* 2003;278(43):41702-8.
19. Visser L, Melief MJ, van Riel D, van Meurs M, Sick EA, Inamura S, et al. Phagocytes containing a disease-promoting Toll-like receptor/Nod ligand are present in the brain during demyelinating disease in primates. *Am J Pathol* 2006;169(5):1671-85.
 20. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124(4):783-801
 21. Song PI, Park YM, Abraham T, Harten B, Zivony A, Neparidze N, et al. Human keratinocytes express functional CD14 and toll-like receptor 4. *J Invest Dermatol* 2002;119(2):424-32.
 22. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, et al.; ALEX Study Team. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J Allergy Clin Immunol* 2004;113(3):482-8.
 23. Ahmad-Nejad P, Mrabet-Dahbi S, Breuer K, Klotz M, Werfel T, Herz U, et al. The toll-like receptor 2 R753Q polymorphism defines a subgroup of patients with atopic dermatitis having severe phenotype. *J Allergy Clin Immunol* 2004;113(3):565-7.
 24. Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, et al. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. *Thorax* 2010;65(8):690-7.
 25. Werner M, Topp R, Wimmer K, Richter K, Bischof W, Wjst M, et al. TLR4 gene variants modify endotoxin effects on asthma. *J Allergy Clin Immunol* 2003;112(2):323-30.
 26. Akdis CA, Akdis M, Bieber T, Bindslev-Jensen C, Boguniewicz M, Eigenmann P, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *Allergy* 2006;61(8):969-87.
 27. Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. *Dermatology* 1993;186(1):23-31.
 28. Skin tests used in type I allergy testing Position paper. Sub-Committee on Skin Tests of the European Academy of Allergology and Clinical Immunology. *Allergy* 1989;44 Suppl 10:1-59.
 29. J. Sambrook, E.F. Fritsch, T. Maniatis. Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y. : Cold Spring Harbor Laboratory, 1982.
 30. StataCorp. Stata Statistical Software: Release 9.0 College Station. TX: Stata Corporation 2001)
 31. Oh DY, Schumann RR, Hamann L, Neumann K, Worm M, Heine G. Association of the toll-like receptor 2 A-16934T promoter polymorphism with severe atopic dermatitis. *Allergy* 2009;64(11):1608-15.
 32. Potaczek DP, Nastalek M, Okumura K, Wojas-Pelc A, Undas A, Nishiyama C, et al. An association of TLR2-16934A>T polymorphism and severity/phenotype of atopic dermatitis. *J Eur Acad Dermatol Venereol* 2010 Jun 20. doi: 10.1111/j.1468-3083.2010.03812.x.
 33. Kormann MS, Ferstl R, Depner M, Klopp N, Spiller S, Illig T, et al. Rare TLR2 mutations reduce TLR2 receptor function and can increase atopy risk. *Allergy*. 2009;64(4):636-42.
 34. Niebuhr M, Langnickel J, Draing C, Renz H, Kapp A, Werfel T. Dysregulation of toll-like receptor-2 (TLR-2)-induced effects in monocytes from patients with atopic dermatitis: impact of the TLR-2 R753Q polymorphism. *Allergy* 2008;63(6):728-34.
 35. Weidinger S, Novak N, Klopp N, Baurecht H, Wagenpfeil S, Rummeler L, Ring J, et al. Lack of association between Toll-like receptor 2 and Toll-like receptor 4 polymorphisms and atopic eczema. *J Allergy Clin Immunol* 2006;118(1):277-9.
 36. Smit LA, Bongers SI, Ruven HJ, Rijkers GT, Wouters IM, Heederik D, et al. Atopy and new-onset asthma in young Danish farmers and CD14, TLR2, and TLR4 genetic polymorphisms: a nested case-control study. *Clin Exp Allergy* 2007;37(11):1602-8.
 37. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999;11(4):443-51.
 38. Mrabet-Dahbi S, Dalpke AH, Niebuhr M, Frey M, Draing C, Brand S, et al. The Toll-like receptor 2 R753Q mutation modifies cytokine production and Toll-like receptor expression in atopic dermatitis. *J Allergy Clin Immunol* 2008;121(4):1013-9.
 39. Williams LK, Ownby DR, Maliarik MJ, Johnson CC. The role of endotoxin and its receptors in allergic disease. *Ann Allergy Asthma Immunol* 2005;94(3):323-32.
 40. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Arch Intern Med* 2002;162(9):1028-32.
 41. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000;25(2):187-91.