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Heterozygous Arg753Gln Polymorphism of Human TLR-2 Impairs Immune Activation by *Borrelia burgdorferi* and Protects from Late Stage Lyme Disease¹

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Lyme disease (LD) is caused by *Borrelia burgdorferi* and displays different stages, including localized, early disseminated, and persistent infection, all of which are associated with profound inflammatory reactions in the host. Induction of proinflammatory cytokines by *B. burgdorferi* is mainly mediated by outer surface proteins interacting with TLR-2/TLR-1 heterodimers. In this study, we show that TNF- α induction by *Borrelia* lysate was impaired in heterozygous TLR-2 knockout mice, while reactivity to lipoteichoic acid, another TLR-2 ligand signaling via TLR-2/TLR-6 heterodimers, was unaffected. Blood from individuals heterozygous for the TLR-2 polymorphism Arg753Gln was tested for cytokine release upon stimulation with *Borrelia* lysate, and induction of TNF- α and IFN- γ was significantly lower as compared with individuals not exhibiting this variation. Overexpression of TLR-2 carrying the Arg753Gln polymorphism in HEK 293 cells led to a significantly stronger impairment of activation by TLR-2/TLR-1 ligands as compared with TLR-2/TLR-6 ligands. To study whether heterozygosity for the Arg753Gln variant of TLR-2 influenced susceptibility for LD, we analyzed 155 patients for this polymorphism. The Arg753Gln variant occurs at a significantly lower frequency in LD patients as compared with matched controls (5.8 vs 13.5%, odds ratio 0.393, 95% confidence interval 0.17–0.89, $p = 0.033$), with an even more pronounced difference when late stage disease was observed (2.3 vs 12.5%, odds ratio 0.163, 95% confidence interval 0.04–0.76, $p = 0.018$). These data suggest that Arg753Gln may protect from the development of late stage LD due to a reduced signaling via TLR-2/TLR-1. *The Journal of Immunology*, 2005, 175: 2534–2540.

Lyme disease (LD)⁶ caused by the spirochete *Borrelia burgdorferi* sensu lato is the most frequent vector-borne disease in the U.S., and is also frequently found in Europe and Northern Asia (1). Incidence increased significantly over the last years, with >23,000 cases in the U.S. in 2002 (2). LD displays different clinical stages: erythema chronicum migrans (ECM) is

associated with early localized infection; early disseminated infection commonly may include facial palsy as well as meningoencephalitis, the latter occurring more frequently in Europe as compared with the U.S. (3). Lyme arthritis is the most common manifestation of late disseminated or late stage LD in the U.S., while acrodermatitis chronica atrophicans (ACA) is more often observed in Europe (4). *B. burgdorferi* sensu lato is composed of three subspecies, including *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*. In the U.S., LD is exclusively caused by *B. burgdorferi*, while in Europe all three subspecies are found (5). *B. afzelii* has been associated with ACA, while *B. garinii* has frequently been isolated from the CSF and is thought to be associated with neurological involvement (6, 7). This hypothesis recently has been strengthened by a mouse model using all three strains resulting in different disease patterns (8).

Immune responses of the host toward *B. burgdorferi* are predominantly mediated by the recognition of outer surface proteins (Osps), which are abundant within these bacteria (9). Osps are anchored within the outer membrane via a triacylated lipid anchor structure comprising a *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*)cysteinyl moiety at the amino terminus, also referred to as Pam₃Cys, shown to induce cytokines in mononuclear phagocytes via TLR-2 in vitro (10–12). To interact with a rather wide spectrum of ligands, TLR-2 forms heterodimers with TLR-1 and TLR-6 (13). Studies using knockout mice identified TLR-1 as the coreceptor required for the recognition of triacylated lipoproteins and lipopeptides (14), while diacylated compounds, including the lipopeptide MALP-2 from *Mycoplasma fermentans* as well as lipoteichoic acid (LTA) from Gram-positive bacteria, interact with TLR-2/TLR-6 heterodimers (14, 15).

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⁶ Abbreviations used in this paper: LD, Lyme disease; ACA, acrodermatitis chronica atrophicans; BMM, bone marrow-derived macrophage; CI, confidence interval; ECM, erythema chronicum migrans; LTA, lipoteichoic acid; Osp, outer surface protein; SNP, single nucleotide polymorphism; wt, wild type.

TLR-2 knockout mice have been shown to be hyporesponsive to vaccination with OspA. In the same study, hyporesponsiveness to vaccination in humans was associated with low expression of TLR-1 (16). Studies on *B. burgdorferi* infection using TLR-2 knockout mice revealed that this receptor is essential for combating this pathogen because knockout mice exhibited elevated bacterial loads in comparison with their wild-type (wt) littermates (17). These data were supported by studies on mice lacking MyD88, a central signaling component of the TLR pathway, which also exhibited a higher pathogen burden after infection (18, 19).

Single nucleotide polymorphisms (SNPs) of TLRs have been repeatedly associated with incidence and course of infectious diseases (reviewed in Ref. 20). For TLR-4, two cosegregating SNPs (Asp299Gly and Thr399Ile) were associated with hyporesponsiveness to inhalation of LPS, the major ligand of TLR-4 (21). In subsequent studies, these SNPs were found to be associated, i.e., with infections caused by Gram-negative bacteria or with periodontitis (22, 23). The Arg677Trp polymorphism of TLR-2 was found to be associated with lepromatous leprosy in a Korean population (24), and with tuberculosis in a Tunisian population (25). However, a recent study provided evidence that a pseudogene present in some populations may have led to false-positive results (26). Among Caucasians, a different SNP, Arg753Gln located within the signal transduction domain of TLR-2, was found to yield a nonfunctional receptor in *in vitro* assays (27, 28) and is present in ~10% of Caucasians (28). One study presented evidence that this polymorphism may be associated with an increased susceptibility to tuberculosis (29), and we have shown recently a correlation with coronary restenosis (30).

Linking data obtained with knockout mice to human disease and disease susceptibility often has the problem that most cases are heterozygous, while knockout animals used for experimentation are homozygous. In this study, we focused on the effect of heterozygosity for TLR-2 on *B. burgdorferi*-mediated cytokine induction. We also investigated whether the Arg753Gln SNP of human TLR-2 is associated with incidence and course of LD in humans. We examined 155 patients suffering from LD displaying a significantly lower frequency of Arg753Gln as compared with matched controls. Our data indicate that the Arg753Gln allele may protect the host from developing clinical symptoms typical for late stage LD.

Materials and Methods

Preparation of *Borrelia lysate*

A total of 300 ml of a *Borrelia* culture (*B. burgdorferi* sensu stricto, strain N40; kindly provided by T. Kamradt, Deutsches Rheumaforschungszentrum, Berlin, Germany) passaged fewer than eight times after isolation from mice was grown to log phase ($\geq 10^8$ /ml) and was washed twice (20 min, 14°C, 10,000 \times g) with pyrogen-free saline solution supplemented with 1 mM MgCl₂. The cell pellet was resuspended in 7.5 ml of saline with 1 mM MgCl₂, and aliquots of 2.5 ml were lysed by sonification (Branson Sonifier model 250/450, 3-mm microtip; Schwäbisch Gmünd). Protein concentration of the lysate was determined with the BSA protein assay (Pierce) following manufacturer's protocol, and protein concentration in the lysate was adjusted to a final concentration of 1 mg/ml with pyrogen-free saline. The lysate preparation contained <0.03 endotoxin units per 10 μ g protein, as assessed by *Limulus* amoebocyte assay (BioWhittaker). Murine bone marrow-derived macrophages (BMMs), human whole blood, and HEK 293 cells (see below) were stimulated with this preparation or with the following stimuli, as indicated: LPS from *Salmonella abortus equi* (Sigma-Aldrich), LTA from *Staphylococcus aureus* (prepared in-house (31)), and triacylated and diacylated lipopeptides (LP3 and LP2, respectively; EMC).

Preparation of BMMs

TLR-2-deficient mice (C57BL/6 background; obtained from The Jackson Laboratory) were crossed with wt mice bred in the animal facilities of the

Burns and Allen Research Institute, Cedars Sinai Medical Center, resulting in TLR-2^{+/-} mice. The animals were maintained under specific pathogen-free conditions. Mice were sacrificed, and femurs and tibias were excised and flushed with DMEM (Mediatech) containing 10% FCS (Invitrogen Life Technologies) and antibiotics/antimycotics (Invitrogen Life Technologies). Bone marrow cells were treated with RBC lysis buffer (eBioscience) and incubated in DMEM/10% FCS containing 10 ng/ml M-CSF (R&D Systems). Medium was changed on days 3 and 5 (10 ng/ml M-CSF at day 3; no M-CSF on day 5). Macrophages were seeded onto 96-well tissue culture plates (5×10^4 cells/well) on days 6–8 and stimulated after 24 h with LPS, LTA, and *Borrelia* lysate, as indicated, for 20 h. Supernatants were stored at -80°C until cytokine measurement.

Stimulation of human whole blood

Human whole blood was freshly drawn from 12 healthy volunteers; 6 of them were heterozygous for the Arg753Gln polymorphism, and 6 had a wt genotype (for genotyping, see below). Differential blood cell counts were performed with a Pentra60 (ABX Technologies) to rule out acute infections. Heparinized blood from each volunteer was diluted 1/5 with RPMI 1640 medium (BioWhittaker) and stimulated for 24 h at 37°C and 5% CO₂. Cell-free supernatants obtained after resuspension and centrifugation at 3000 \times g for 2 min were stored at -80°C until cytokine measurement.

Cytokine measurement by ELISA

The concentrations of human TNF- α and IFN- γ in the supernatants were measured by in-house sandwich ELISA using commercially available Ab pairs from Pierce (Perbio Science) for TNF- α and IFN- γ . rTNF- α (a gift from S. Poole, National Institute for Biological Standards and Controls, Herts, U.K.) and rIFN- γ (purchased from Thomae) were used as standards. Murine TNF- α release was determined with an ELISA kit from eBiosciences.

Culture, transfection, and stimulation of HEK 293 cells

HEK 293 cells were cultured in DMEM medium (Invitrogen Life Technologies), including sodium pyruvate, and supplemented with 10% FBS, 10 kU/ml penicillin, 10 g/ml streptomycin, and 200 mM L-glutamine. For stimulation experiments, cells were cultured at a density of 1×10^5 cells/well in 12-well tissue culture plates overnight. Transfection with expression plasmids encoding β -galactosidase (0.04 μ g) and the ELAM NF- κ B luciferase reporter plasmid (0.12 μ g; both kindly provided by C. Kirschning, Technische Universität, Munich, Germany) for 24 h was performed using 0.5 μ l/well Fugene (Roche). Cells were additionally transfected with human TLR-2 (C. Kirschning). For some experiments, the same plasmid exhibiting the polymorphism Arg753Gln, inserted with the QuikChange Site-Directed Mutagenesis Kit (Stratagene), was used. Cells were stimulated with LP2 and LP3 in 0.5 ml of DMEM medium without FCS for 20 h, followed by lysis and measurement of β -galactosidase and luciferase activity using a kit based on chemiluminescence (Roche).

Patient study population

Within this study, 155 patients were included displaying symptoms consistent with LD as well as positive testing in ELISA and Western blotting. Informed consent was obtained from all individuals included in this study. The ethics committee approved the study, and it was performed in concordance with the clinical research guidelines of the Charité Medical Center. Patients were stratified according to the case definitions introduced by the European Union Concerted Action on Lyme Borreliosis (www.vie-dis.strath.ac.uk/vie/LymeEU). Patients suffering from malignant disease or individuals displaying occupational risk factors for LD, such as, i.e., forest workers, were excluded from the study. Healthy volunteers from Berlin, Germany ($n = 322$), and Constance, Germany ($n = 27$), were examined as controls. Samples were shuffled randomly and matched with patient samples regarding gender and age (≤ 30 , 31–50, and ≥ 51 years). DNA was extracted from EDTA blood using the Qiamp DNA blood mini kit (Qiagen).

Estimation of Arg753Gln via restriction fragment length polymorphism

Presence of the Arg753Gln polymorphism was estimated according to a previously published protocol (28). In brief, a 340-bp fragment was amplified using Qiagen TaqDNA polymerase and a deoxyribonucleoside triphosphate mix (BD Clontech) in a total volume of 25 μ l on a Biometra Trio-block. Conditions: 95°C for 10 min, 35 cycles of 95°C/30 s, 58°C/30 s, and 72°C/25 s, followed by 72°C for 5 min. PCR products (4 μ l) were digested with *AclI* (0.125 U/sample) at 37°C overnight, followed by electrophoresis on 1% agarose gels (Roth) and 0.75% Nusieve (Biozym).

Statistics

For in vitro experiments, an unpaired *t* test was used when comparing two groups. For comparison of more than two groups, repeated measure ANOVA was instrumental, followed by Bonferroni's multiple comparison test using GraphPad Prism. Cytokine data are expressed as means \pm SEM of 10 mice or 6 blood donors, respectively. All patient SNP analyses were analyzed using the two-tailed Fisher exact test and odds ratio with 95% confidence interval (CI). Values of *p* of 0.05 and lower were considered significant.

Results

To analyze whether one allele of a nonfunctional TLR-2 impairs in vitro cytokine responsiveness to *Borrelia* lysate, we examined the responsiveness of BMMs from TLR-2 wt, TLR-2^{+/-}, and TLR-2^{-/-} mice. BMM were stimulated with LPS, LTA, or *Borrelia* lysate. All cells responded uniformly to the TLR-4 agonist LPS (Fig. 1). As expected, cells from TLR2^{-/-} mice showed an impaired response to LTA and *Borrelia* lysate (Fig. 1). BMM from TLR2^{+/-} mice did not differ from wt cells when stimulated with LTA; however, there was a significant difference in TNF- α levels when *Borrelia* lysate was used as a stimulus.

To investigate whether one nonfunctional TLR-2 allele also affects cytokine induction by *Borrelia* lysate in humans, we performed stimulation assays with whole blood from volunteers. Healthy volunteers were characterized for the presence of Arg753Gln by restriction fragment length polymorphism analysis, and six donors displaying this variant in a heterozygous state were compared with six donors without this SNP. Whole blood was stimulated with *Borrelia* lysate, and comparable to the experiments with murine cells, subjects heterozygous for Arg753Gln showed a partially impaired TNF- α and IFN- γ response toward *Borrelia* lysate (Fig. 2). The differences between the two groups tested persisted when differential cell counts were included in the analysis. Accordingly, a heterozygous state of TLR-2 impairs the response to *Borrelia* lysate, both in cells from heterozygous mice and from humans heterozygous for Arg753Gln. This is in contrast to previous stimulation experiments with LTA, in which a heterozygous state did not affect cytokine induction (32).

A potential explanation for this finding is that a nonfunctional TLR-2 in a heterozygous state may preferentially influence signaling via TLR-2/TLR-1 heterodimers, such as triacylated lipoproteins found in *Borrelia*, while signaling initiated by TLR-2/TLR-6 ligands, such as diacylated lipopeptides or LTA, is less affected. To test this hypothesis, we simulated the heterozygous state in

vitro by transfecting HEK 293 cells with the cDNA encoding for wt TLR-2, the TLR-2 carrying the Arg753Gln mutation, or a 50:50 mixture of the two expression plasmids. The cells were then stimulated with Pam₂Cys, a diacylated lipopeptide, or with Pam₃Cys, a triacylated lipopeptide corresponding to *Borrelia* Osp. Additionally, *Borrelia* lysate was tested. We have shown previously that in these cells Pam₂Cys uses TLR-2/TLR-6 heterodimers, while Pam₃Cys mainly stimulates via TLR-2/TLR-1 (33).

The heterozygous receptor led to an only slightly changed reduction in Pam₂Cys-induced activity, while Pam₃Cys and the *Borrelia* lysate were reduced in their stimulatory capacity to ~50% as compared with the wt receptor (Fig. 3A). Transfection of the mutated TLR-2 exclusively led to a residual activity of ~30% when Pam₂Cys was used as stimulus, while Pam₃Cys and *Borrelia* lysate almost failed to display any activity. For both experiments, with the heterozygous receptor transfected and the mutated variant only, the difference observed between TLR-2/TLR-6 and TLR-2/TLR-1 ligand stimulation was statistically significant. These results were confirmed by transfecting a mixture of wt and mutated TLR, with increasing percentages of the mutant TLR-2 (Fig. 3B). In this study, LP2 led to an increase in luciferase activity even when the transfection contained only 3.125% of wt TLR-2, while for *Borrelia* lysate, at least 25% of wt TLR-2 was needed to result in an enhanced activation (Fig. 3B).

To investigate whether TLR-2 may influence susceptibility to LD, patients diagnosed for LD were screened for the presence of the Arg753Gln variant. We investigated 155 individuals suffering from LD (Table I). As controls, 349 German Caucasians with no history of LD were analyzed. Among the patients, 27 showed typical signs of ECM and were therefore classified as early localized or stage I disease. We identified 88 patients with late disseminated or stage III LD, the majority suffering from Lyme arthritis (*n* = 68), while 15 patients showed clinical signs of ACA (*n* = 15). Patients were age and gender matched, and frequencies of the Arg753Gln SNP were determined. We failed to detect any individual homozygous for this polymorphism, which is in line with Hardy Weinberg equilibrium (*p* = 0.062, using a two-sided Fisher's exact test for controls). The following analysis refers to heterozygous individuals instead of allelic frequencies.

Of the 155 patients investigated, 9 individuals were heterozygous for the Arg753Gln polymorphism (Table II). As compared with all controls (*n* = 349) as well as with the age- and gender-matched control group (*n* = 155), these data indicate a significantly lower frequency of this SNP among the patients. We next addressed whether this trend was present at all clinical stages represented among the patients. For patients with clinical signs of ECM, there also was a trend toward lower frequency of Arg753Gln because no SNP was detected. Finally, we stratified the results for patients with late disseminated or stage III disease, the majority suffering from LA: in this study, the frequency of individuals carrying the Arg753Gln SNP was significantly lower as compared with the controls (*p* = 0.005, odds ratio 0.17, 95% CI 0.04–0.72 for all controls; *p* = 0.003, odds ratio 0.15, 95% CI 0.03–0.65 for matched controls). Thus, the deviation from the control group was stronger as compared with the complete LD group, and persisted when we compared stage III patients with 88 matched controls.

Discussion

Innate immune recognition of *B. burgdorferi* is a hallmark in the development of disease, as shown by a series of studies using knockout mice. In homozygous TLR-2-deficient mice, higher levels of spirochetes were found in tissues following infection as compared with the wt littermates supporting the role of TLR-2 in

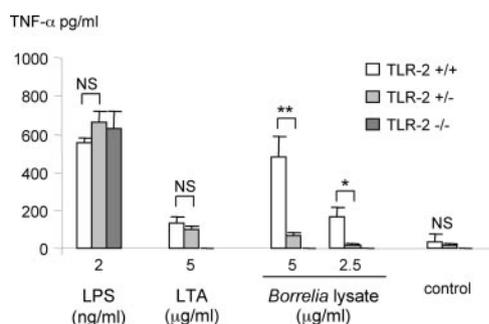
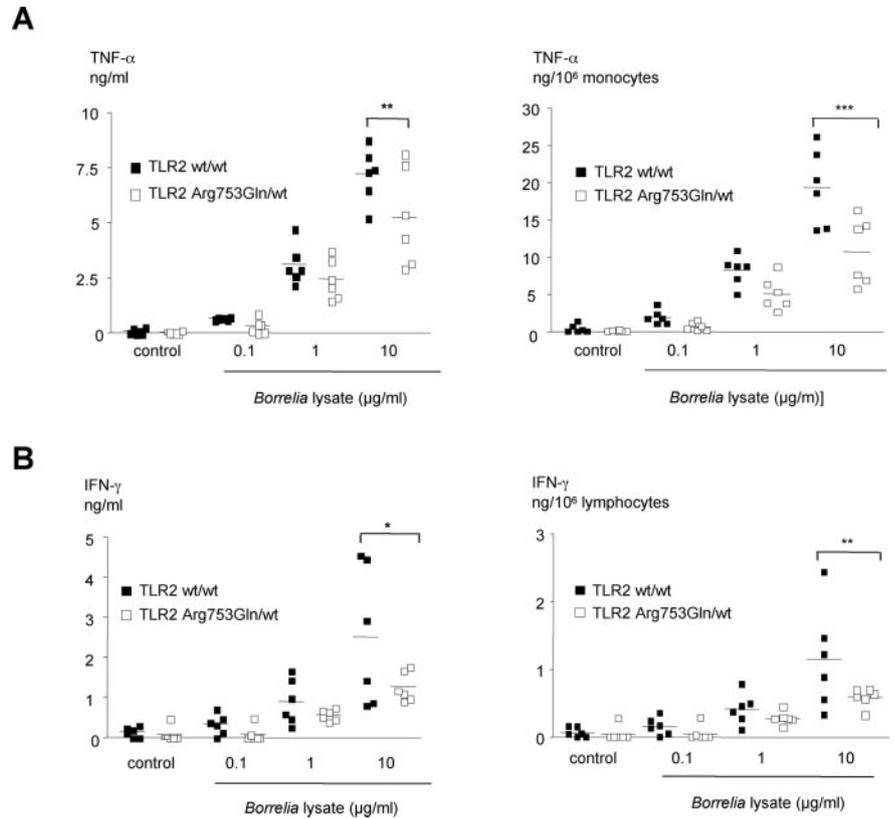


FIGURE 1. BMM from TLR2^{+/-} mice show reduced cytokine release in response to *B. burgdorferi*. BMMs from TLR2^{+/+}, TLR2^{+/-}, and TLR2^{-/-} mice were incubated for 20 h in 96-well culture plates in the presence of 2 ng/ml LPS, 5 μ g/ml LTA, as well as 5 and 2.5 μ g/ml *Borrelia* lysate. TNF- α in the supernatant was determined by ELISA. Data are presented as means \pm SEM of three measurements; shown is one of three independent experiments with similar results. *, *p* < 0.05; **, *p* < 0.01 by Student's *t* test.

FIGURE 2. Heterozygous Arg753Gln polymorphism leads to reduced *Borrelia*-induced TNF- α and IFN- γ levels in whole blood. Heparinized blood from six subjects with the heterozygous polymorphism and six wt controls was stimulated under blinded conditions for 24 h in the presence of 0.1, 1, and 10 $\mu\text{g/ml}$ *Borrelia* lysate or left untreated (control). TNF- α (A) and IFN- γ (B) in the supernatant were determined by ELISA. Data are presented as nanograms per milliliter (left column) and calculated per 10^6 monocytes (for TNF- α) or per 10^6 lymphocytes (for IFN- γ , right column). Data are representative for two independent experiments and are presented as means \pm SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs wt controls as tested by Bonferroni's multiple comparison test.



combating these bacteria (17), and studies investigating homozygous MyD88 knockout mice came to similar conclusions (18, 19). In this study, we aimed at investigating the impact of a single nonfunctional allele of TLR-2 on innate immune recognition of *B. burgdorferi* by studying in vitro responses to *Borrelia* lysate as well as the distribution of the Arg753Gln SNP among patients suffering from LD. The lack of one functional allele of TLR-2

impairs cellular recognition of this pathogen, as experiments with BMMs from heterozygous mice revealed a significantly decreased cytokine release upon stimulation with *Borrelia* lysate. Interestingly, there was no difference when LTA was tested, indicating that triacylated amphiphilic compounds, such as Osps found in *Borrelia* spp., are more affected by alterations of TLR-2 as compared with diacylated compounds.

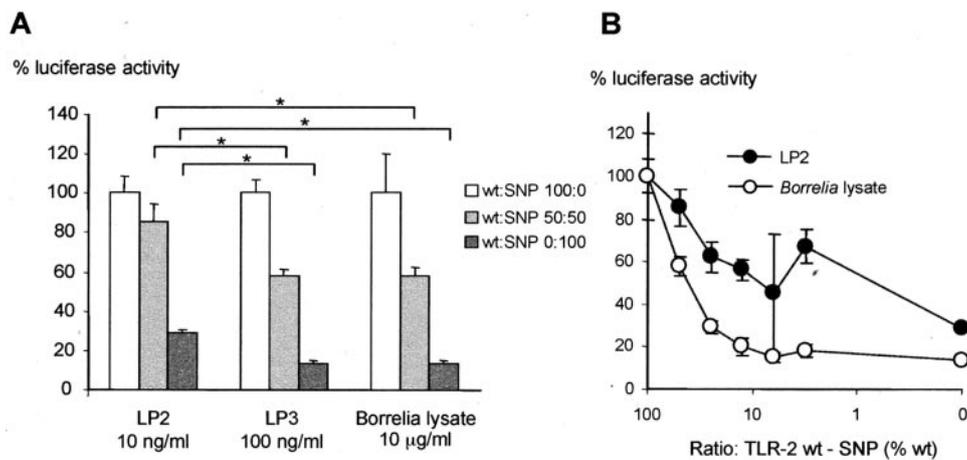


FIGURE 3. Effect of transfection of HEK 293 cells with TLR-2 as well as TLR-2 carrying the Arg753Gln variant. HEK 293 cells were cultured at a density of 100,000/well in 12-well tissue culture plates in DMEM + 10% FCS overnight, followed by transfection with a plasmid encoding for β -galactosidase, an ELAM NF- κ B reporter plasmid, as well as plasmids encoding for wt human TLR-2 or human TLR-2 with the inserted Arg753Gln mutation, as indicated, using FuGENE 6. A, Cells were transfected with wt TLR-2 only, a 1:1 mixture of wt and mutant TLR-2, or mutant TLR-2 only. Twenty-four hours after transfection, cells were stimulated with 10 ng/ml LP2, 100 ng/ml LP3, or 10 $\mu\text{g/ml}$ *Borrelia* lysate for 20 h, followed by estimation of cellular activation using chemiluminescence. Experiments were performed in triplicates; shown are representatives of three separate experiments. B, Cells were transfected with wt TLR-2 and mutant TLR-2 at the following ratios: 100:0, 50:50, 25:75, 12.5:87.5, 6.25:93.75, or 3.125:97.875. Twenty-four hours later, cells were stimulated with 10 ng/ml LP2 or 10 $\mu\text{g/ml}$ *Borrelia* lysate for 20 h, followed by measurement of chemiluminescence. Experiments were performed in triplicates; shown are representatives of two separate experiments. Statistics were performed using Student's t test; p values < 0.01 are indicated by two asterisks.

Table I. Composition of the patient group

	Cases			Matched Groups			All Controls		
	<i>n</i>	Mean age	% Male	<i>n</i>	Mean age	% Male	<i>n</i>	Mean age	% Male
Total	155	49.7 ± 14.2	54.8	155	50.9 ± 15.9	54.8	349	55.6 ± 18.1	44.4
Stage I	27	47.1 ± 14.2	63.0	27	52.1 ± 14.3	63.0			
ECM	27	47.1 ± 14.2	63.0	27	51.4 ± 15.5	63.0			
Stage II	40	51.7 ± 15.2	65.0	40	49.0 ± 15.5	65.0			
ME ^a	36	51.2 ± 15.9	61.1	36	48.3 ± 16.0	61.1			
MC	2	54.5 ± 5.5	100	2	51.5 ± 10.5	100			
other	2	58.0 ± 2.0	100	2	58.5 ± 1.5	100			
Stage III	88	49.0 ± 13.6	47.7	88	51.3 ± 16.6	47.7			
LA	68	46.3 ± 13.7	50.0	68	47.8 ± 16.5	50.0			
ACA	15	57.6 ± 6.0	33.3	15	64.9 ± 10.1	33.3			
other	5	59.0 ± 11.3	60.0	5	58.0 ± 6.9	60.0			

^a ME, meningoenophalitis; MC, myocarditis; LA, Lyme arthritis.

To examine the role of the heterozygous Arg753Gln TLR-2 SNP, a model of human whole blood incubation was used allowing for monitoring of monocyte and lymphocyte function within a physiological environment (34–36). In line with the observations in heterozygous TLR-2 knockout mice, we saw lower levels of both TNF- α and IFN- γ when blood from donors heterozygous for Arg753Gln was stimulated with *Borrelia* lysate as compared with donors without this variation. This finding is in contrast to our previous studies on the impact of the Asp299Gly SNP of TLR-4 as well as Arg753Gln on stimulation by LPS or different LTA preparations, respectively (32, 37). Thus, innate immune recognition of triacylated lipoproteins as found in *Borrelia* spp. is apparently more sensitive to variations in TLR-2 expression as compared with TLR-4 ligands and diacylated TLR-2 ligands. We were able to strengthen this hypothesis by an in vitro overexpression model, in which stimulation with triacylated lipopeptide and *Borrelia* lysate was more strongly affected by heterozygous TLR-2 expression as compared with stimulation with a diacylated lipopeptide. One potential explanation for this difference may be that the heterozygous expression of a nonfunctional TLR-2 as analyzed in this study may affect the TLR-2/TLR-1 heterodimer formation more severely as compared with the TLR-2/TLR-6 heterodimer formation. It has to be noted that throughout this study we used a lysate derived from *B. burgdorferi* sensu stricto. Although we cannot rule out that lysates from other pathogenic species such as *B. garinii* or *B. afzelii* may display differential effects, this does not appear to be likely because our data indicate that the major active compounds in these

preparation are Osp, and these lipoproteins are abundant in *B. garinii* and *B. afzelii* as well (38).

Our genotyping analysis shows that the Arg753Gln SNP occurs at a lower rate in patients suffering from LD as compared with matched controls, and this was highly significant in late stage LD patients. Our data thus suggest that an impaired recognition of *B. burgdorferi* due to a nonfunctional TLR-2 may protect from persistent disease. One explanation for this finding is that clinical symptoms of late stage LD are mediated by inflammatory mediators, i.e., cytokines, such as TNF- α and IFN- γ studied in this work. Triggering of TLR-2 by *Borrelia* Osps induces these proinflammatory cytokines both playing an important role in the development of arthritis (39), and it has been proposed that arthritis may be caused by Osp-specific Th cells cross-reacting with human LFA-1 (40). As we show in this work, heterozygosity for the Arg753Gln TLR-2 SNP impairs the release of TNF- α and IFN- γ . We suggest that this may be beneficial for the host in the context of late stage disease because arthritogenic effects of cytokines are less pronounced, while bacteria can still be eliminated. It has to be noted that we also observed a trend toward a lower frequency of the Arg753Gln SNP among the small subgroup of patients with ECM, which was, however, not significant. Due to the small sample size, we cannot rule out that development of ECM may also be influenced by TLR-2 SNPs, and future studies are needed to clearly define the role of TLR-2 variants in acute LD. Two recent studies investigating whether the Arg753Gln polymorphism affects the clinical course of an infectious disease came to controversial

Table II. Frequency of the Arg753Gln SNP among patients suffering from LD

Patients	Arg753Gln <i>n</i> (%)	All Controls ^a			Matched Controls			Matched Groups		
		<i>n</i> (%)	OR ^b 95% CI	<i>p</i> ^c	<i>n</i> (%)	OR 95% CI	<i>p</i> Value	<i>n</i> (%)	OR 95% CI	<i>p</i>
Total (<i>n</i> = 155)	9 (5.8)	42 (12.0)	0.459 0.21–0.95	0.0369	21 (13.5)	0.393 0.17–0.89	0.0331	ND	ND	ND
Stage I (<i>n</i> = 27)	0 (0)	42 (12.0)	0.132 0.01–2.19	0.0570	21 (13.5)	0.114 0.01–2.24	0.0835	4 (14.8)	0.095 0.01–1.86	0.1110
Stage II (<i>n</i> = 40)	7 (17.5)	42 (12.0)	1.551 0.64–3.73	0.317	21 (13.5)	1.354 0.53–3.45	0.6127	6 (15)	1.202 0.37–3.96	1.0
Stage III (<i>n</i> = 88)	2 (2.3)	42 (12.0)	0.170 0.04–0.72	0.0047	21 (13.5)	0.148 0.03–0.65	0.0028	11 (12.5)	0.163 0.04–0.76	0.0179

^a Population size, *n* = 349.

^b All statistics were performed with GraphPad Prism software.

^c Values of *p* were determined using a two-sided Fisher exact test. Significant results are printed in bold.

results: while one study, in line with our results, showed an altered susceptibility to pulmonary tuberculosis among carriers of this variant (29), another study investigating infections caused by the Gram-positive pathogen *S. aureus* did not reveal any association (41). These data support our findings that this SNP affects the recognition of triacylated, but not diacylated stimuli, because triacylated lipoproteins were described in *Mycobacterium tuberculosis* (42, 43), while LTA from *S. aureus* is diacylated (31).

Our finding that Arg753Gln protects from clinical symptoms of late stage infection with *B. burgdorferi*, including Lyme arthritis, appears to be in contrast to studies investigating TLR-2 and MyD88 knockout mice, because these animals displayed more severe arthritis than the corresponding wt mice (17–19, 44), and mice heterozygous for TLR-2 did not differ from wt mice (17). However, arthritis in mice experimentally infected with *B. burgdorferi* is considered to be a subacute stage of disease, comparable to meningoencephalitis or myocarditis in humans, in which bacteria are present in the tissues, and we did not observe any association of subacute stages of LD with TLR-2 SNPs. Mice do not develop persistent infection comparable to late stage LD observed in humans (45), and the pathology observed in humans during late stage diseases is thought to be mainly caused by *B. burgdorferi*-specific T cells secreting cytokines, especially IFN- γ (4, 45). The reason for this discrepancy may be related to the similarities between *Borrelia* Osps and human LFA-1, as mentioned above (40). The causative role of IFN- γ production for the development of arthritis in LD is in line with our hypothesis that lower levels of this and other cytokines protect from late stage LD, while acute and subacute stages are not affected. Furthermore, one study showed that, while arthritis was enhanced in TLR-deficient mice, carditis was less severe, indicating that an impaired recognition of *B. burgdorferi* may also have beneficial effects on the course of infection in these animals (44).

There is growing evidence that SNPs of innate immune recognition molecules may have an impact on incidence and course of infectious diseases (22, 24, 25, 29). One other study came to the conclusion that SNPs within a gene encoding for a TLR may also protect from disease: in this study, atherosclerosis, a chronic inflammatory disease, was found to be associated with a lower frequency of SNPs of TLR-4 (46). The hypothesis, that SNPs within genes encoding for TLRs may protect from chronic inflammatory diseases, could potentially explain the sustained high prevalence of these SNPs among Caucasians (28). Further prospective studies investigating both cytokine patterns of patient-derived leukocytes and clinical outcome should be performed to verify this hypothesis.

This is the first study that links protection toward late stage LD to a TLR-2 polymorphism. In line with other recent studies, it further supports the key role of the innate immune system and in particular the TLRs as critical determinates of the initiation and course of infection and inflammation. We conclude that together with a timely identification of bacteria, studying the host's genetic disposition may have a profound effect on prophylactic and therapeutic interventions in LD.

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Disclosures

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