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Toll-Like Receptor 2 (P631H) Mutant Impairs Membrane Internalization and is a Dominant Negative Allele

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Abstract

We have sequenced 416 Toll-like receptor-2 (TLR2) alleles in 208 subjects in a tuberculosis case–control study in Croatian Caucasian population. We found ten single nucleotide polymorphisms (SNP) among which three were novel (S97S, T138I and L266F). The genotype containing TLR2-P631H SNP was significantly overrepresented in patients with tuberculosis when compared to contact controls, suggesting a small yet increased risk to disease. The causative agent of tuberculosis is *Mycobacterium tuberculosis,* which can bind to TLR2 with its lipoprotein coat. The TLR2-P631H mutant has a dominant negative effect on the wild type TLR2 signalling in transfected HEK293 kidney cells using the NF-κB-driven luciferase as a reporter gene with ligands like *M. avium* extracts, Pam3CysSK4 or FSL-1 that bind TLR2/TLR1 or TLR2/TLR6 heterodimers, respectively. Studies on internalization from the Regular Madine Darby Canine Kidney cell surface into the early endosomal compartments showed a lower rate of the mutant compared to the wild type. Our data, in combination with a report by others show that the TLR2-P631H allele could be associated with protection to meningococcal meningitis, suggest that by dominantly inhibiting the response of cells important in the immune response this mutant might confer either protection or susceptibility to meningitis or tuberculosis, respectively.

Introduction

Toll-like receptors (TLR) are transmembrane molecules utilized by cells of the innate immunity as sentries for pathogenicity, being a kind of evolutionary memory of molecular patterns associated with past infections. Thirteen mammalian TLR, TLR1-13, have been identified to date [1]; however, only ten functional ones were found in humans. Each TLR has a different set of ligand specificities that are rare or absent in vertebrates. Bacterial components are mainly recognized by cells having cell-surface TLR; i.e. Gram-positive bacteria, lipopeptides and lipoteichoic acid (LTA) are bound by TLR2 in co-operation with TLR1 or TLR6 [2–8]. Lipopolysaccharide from Gram-negative bacteria is recognized by cells bearing TLR4 complexed with the small-secreted glycoprotein MD-2. Flagellin binds to TLR5. The endosomally localized TLR, TLR3, TLR7/8 and TLR9 bind viral components, i.e. TLR3 can bind double-stranded RNA, TLR7 and TLR8 single-stranded RNA, and TLR9 can bind hypomethylated stretches of CpG-rich DNA, commonly found in bacteria and viruses (reviewed in Barton [9], Dembic [10] and Kawai 2005 [1]).

The intracellular signalling is regulated by the intracytosolic domain of TLR that shares homology with the IL-1 receptor called Toll/IL-1 receptor (TIR) domain. This region can activate downstream signalling pathways leading to IRF3 and/or NF-κB activation [9]. Upon binding a ligand, TLR2 recruits TIRAP and myeloid differentiation factor 88 (MyD88) leading to NF-κB activation. Cell surface trafficking and internalization of TLR2 has recently been studied in greater detail. For these and similar studies of the underlying cell-biological events in the future, TLR2 functional mutants would be of great help in deciphering its physiological function.

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The TLR2 heterodimer, in combination with the TLR1, binds 19-kD mycobacterial lipoprotein [11], suggesting it is an adhesion molecule for bacillus *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Worldwide, tuberculosis is the greatest killer among infectious diseases, with yearly deaths rising in millions albeit mostly in underdeveloped countries. However, recently it poses a great danger for health in developed countries too, because of increased number of diseased with antibiotic-resistant tuberculosis. *M. tuberculosis* causes disease in 5–10% of infected persons, provided no immunodeficiencies were present. This is probably because of complex inheritance of predisposing factors that are yet to be understood, which fuels ongoing extensive genetic research.

Previous studies linked the TLR2 variants R753Q and R677W with predisposition to tuberculosis in Turkey [12] and Tunisia [13], respectively, despite a low number of subjects in the studies. A variant of the TLR signalling molecule TIRAP (S180L) was also associated with tuberculosis [14–16]. Likewise, the TLR2 genotype 597CC was associated with susceptibility to meningitis caused by *M. tuberculosis* in a Vietnamese population [17].

For these reasons, we sought to identify naturally occurring TLR2 mutants in a case–control study on tuberculosis in Croatia and to explore their eventual association with disease.

Materials and methods

Patients and controls. Patients with tuberculosis were stationed and cured at the clinic in Rijeka, Croatia, where the initial diagnosis was made as previously reported [18–20]. In short, medical assessment of patients was made clinically at the Department of Pulmology, Clinic for Internal Medicine, Clinical Hospital Center Rijeka, and supported by identification of *M. tuberculosis* from sputum by *in vitro* culturing procedure. All patients (n = 247) and controls (n = 517) provided oral and written informed consent. The mean (±SD) age for patients was 51.03 (±15.71) years, and 77.59% were men. The number of men predominates the patient database and reflects the actual sex distribution of patients admitted at the Clinic of Internal Medicine (Section of Pulmology) in Rijeka during sample collection. The contact controls (n = 85) were a) employees at the Section of Pulmology because we assumed they were in contact with the *M. tuberculosis* during their work with the patients and b) patients' household healthy members. None of them had familial relation to the diseased. The other healthy control subjects were unrelated blood donors. The blood was donated to the Department of Transfusion Medicine, the Clinical Hospital Center Rijeka. Control persons were mostly men (60.80%) with a mean age of 41.84 ± 11.90(SD) years. The patient and control groups were matched by ethnicity (mostly Croatian Caucasian), age and socioeconomic status, and closely matched by sex, which was off by 17%. Thus, we believe that putative confounding and causal variables were equally distributed among case and control groups.

To ascertain the protection of human subjects in research, the ethics committee of the Medical Research Council at the Medical faculty at the University of Rijeka approved the study.

In vitro DNA amplification and sequencing. TLR2 gene-specific DNA fragments were amplified from human genomic DNA using various TLR2-specific primers (Table 1). For polymerase chain reaction (PCR) amplification of the coding sequence of TLR2 fragments for sequencing, the 20-μL PCR mixtures contained the following: 0.2 μM each of the specific primers, 0.2 mM deoxyribonucleotide triphosphate (dNTPs), 1 × Taq buffer with KCl (10 mM Tris–HCl, 50 mM KCl, 0.08% Nonidet P40) (pH 8.8 at 25 °C), 1.5 mM $MgCl_2$ and 0.6 U of Taq polymerase (Fermentas Life Sciences, Ontario, Canada) and 20 ng of genomic DNA. The entire coding region of TLR2 was amplified by sequential amplification of various fragments sizes using the primer combination described in Table 1. Temperature cycling was performed in Peltier Thermal cycler (MJ Research, Massachusets, USA) as follows: 95 °C denaturizing for 20 s, 1 min annealing at the specified annealing temperature (Table 1) for the respective primer combination and 2 min extension at 72 °C for 35 cycles; followed by a final extension at 72 °C for 8 min. Amplicons were visualized by ethidium bromide-stained agarose gel electrophoresis using the Geldoc imaging system (Bio-Rad, Hercules, CA, USA); purified by ExoSAP-IT® For PCR Product Clean-Up protocol (USB, Ohio, USA) and quantified by the NanoDrop®ND-1000 (Nanodrop Technologies, Wilmington, USA) protocol. Purified amplicons were sequenced on 3130 × l Genetic Analyzer automatic sequencer using the Big Dye 3.1v chemistry (Applied Biosytems, CA, USA) using the primers described in Table 1. Sequences were analysed using the Sequencher™ software (Genes Codes Corporation, Ann Arbor, MI, USA).

Single nucleotide polymorphism (SNP) and statistical analyses. The typing of three TLR2 SNP at T597C (N199N), T1350C (S450S) and C1892A (P631H) was performed by allele discrimination Taqman-based PCR assay. Listed in Table 1 are the primers and probes for these assays, which were purchased from the Applied Biosystems (ABI, Foster City, CA, USA). The assays were performed in Mx4000 real time PCR apparatus (Stratagene, La Jolla, CA, USA).

Statistical analyses listed in Tables 2, 3 and 4 were carried out by the Chi-square test using the software program Statcalc (Acastat Software, Leesburg, VA, USA). Phase software program [21, 22] was used to reconstruct

Table 1 TLR2 gene: PCR amplification and DNA sequencing primers.

Forward (F)/Reverse (R) DNA primers and probes	(5′-3′)	Location[a]	Length (bp)	PCR-generated fragment (bp)	Application (annealing temperatures °C)
TLR2-EX2C-F	ACGATATGCTGTCAAACACA	−66–(−47)	20	944	PCR/Sequencing (55/52)
TLR2-EX2C-R	CCAACTCCATTAAGGGTACA	859–878	20		
TLR2-EX2B/C-F	GAGAGTGGGAAATATGGACA	462–481	20	885	PCR/Sequencing (55/52)
TLR2-EX2B/C-R	TGTATTCGTGTGCTGGATAA	1327–1346	20		
TLR2-EX2B- F	TGTACCCTTAATGGAGTTGG	859–878	20	911	PCR/Sequencing (55/52)
TLR2-EX2B- R	AGTGCTGTCCTGTGACATTC	1750–1769	20		
TLR2-EX2A/B-F	TTATCCAGCACACGAATACA	1327–1346	20	889	PCR/Sequencing (55/52)
TLR2-EX2A/B-R	GCTCCAGAAGAATGAGAATG	2196–2215	20		
TLR2-EX2A-F	CTTCATTTGCTCCTGTGAAT	1602–1621	20	871	PCR/Sequencing (55/52)
TLR2-EX2A-R	ACGGTACATCCACGTAGTTT	2441–2460	20		
TLR2-KpnI-F2*	gggtggtaccATGCCACATACTTTGTGGAT	1–20	30	2375	Genomic PCR (/50)
TLR2-XhoI-R*	ggtctcgagcCTAGGACTTTATCGCAGCTC	2335–2355	30		
TLR2-FL-F*	caccATGCCACATACTTTGTGGAT	1–20	24	2359	Genomic PCR (/50)
TLR2-FL-R	CTAGGACTTTATCGCAGCTC	2335–2355	20		
TLR2 N199N:					
TLR2-T597C-F	CAGATCTACAGAGCTATGAGCCAAAA	551–576	26	97	Allele discrimination
TLR2-T597C-R	TCTCCAGCAGTAAAATATGCTGCTT	622–646	25		PCR (/60)
T597C (C) FAM-probe	CAGATGACTTACGTTCTGA	591–609	19		
T597C (T) VIC-probe	TCAGATGACTTACATTCTGA	591–610	20		
TLR2 S450S:					
TLR2-T1350C -F	GAAATATTTGAACTTATCCAGCACACGAA	1314–1344	29	86	Allele discrimination
TLR2-T1350C -R	GTTGCTAACATCTAAAATTTCCAGTGTCTT	1368–1398	30		PCR (/60)
T1350C (C) FAM-probe	AGCCTGTTACGCTGTGT	1344–1360	17		
T1350C (T) VIC-probe	CAGCCTGTTACACTGTGT	1344–1361	18		
TLR2 P631H:					
TLR2-P631H-F	GGCCTGTGGTATATGAAAATGATGTG	1840–1865	26	106	Allele discrimination
TLR2-P631H-R	CACTGTAAGAAACAAATGCATCATAGCA	1918–1945	28		PCR (/60)
C1892A (A) FAM-probe	CTTTCCTGTGCTTCCT	1885–1900	16		
C1892A (C) VIC-probe	TTTCCTGGGCTTCCT	1885–1899	15		

Reference sequence: Ensembl TLR2 Transcript ID #ENST00000260010.
*Nucleotides in lower case denote non-genomic DNA additions to primers.
[a]+1 is the A of the translation start codon.

haplotypes from population genotype data obtained by typing these three SNP (Table 4). Population statistics, Hardy–Weinberg equilibrium and linkage disequilibrium analyses were carried out using Arlequin ver 3.1 software (Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland). All PCR-typed SNP loci were in Hardy–Weinberg equilibrium in three tested populations: patients (n = 247), contact controls (n = 85) and (combined contact and healthy blood donor) controls (n = 517), except the N199N locus, which was not in the latter control group. The contact control population was not significantly different in size compared to the patient population. Linkage disequilibrium was found between the first two (N199N, S450S) loci in all populations, and also between the first and the third locus (N199N, P631H) in patient and control populations. However, there was only a trend towards linkage disequilibrium between the second (S450S) and the third (P631H) locus. Regarding correction for multiple comparisons, when typed loci are in linkage disequilibrium, testing one is nearly the same as testing another. For this reason, a strict Bonferroni correction by numbers of markers is not reasonable. A conservative adjustment would allow for multiplying our reported P-values, but only by the loci not in the linkage equilibrium.

Gene expression vectors and constructs for cell transfections. The coding region of the TLR2 gene was amplified by PCR from human genomic DNA. For cloning the P631H mutant of TLR2, a 2375-bp TLR2 gene DNA fragment was amplified from 100 ng genomic DNA of patient with tuberculosis having the P631H mutation, using TLR2-KpnI-F and TLR2-XhoI-R primer pairs (Table 1) and the PfuUltra™ High-Fidelity DNA Polymerase (Stratagene, CA, USA). The generated fragment was cloned into Zero Blunt® PCR Cloning Plasmid by transforming TOPO10 chemically competent bacteria (Invitrogen Corporation, CA, USA). The presence of the P631H mutation in the recombinant plasmids was verified by allele discrimination PCR using the TLR2-P631H-F/TLR2-P631H-R primers, TLR2-P631H FAM and VIC probes (Table 1) in the Mx4000 apparatus (Stratagene).

In the next step, P631H positive recombinant plasmid was doubly digested with KpnI and XhoI endonucleases (Fermentas Life Sciences, Ontario, Canada) and the resulting 2375-bp DNA fragment was subcloned into the

Table 2 Association analysis of TLR2 SNP obtained by sequencing genomic DNA. Summary of comparison of TLR2 mutations observed in Caucasian subjects from Croatia with tuberculosis (n = 103 individuals and 515 kb examined) and normal controls (n = 105 individuals and 525 kb examined), which included contact controls (n = 83 individuals and 415 kb examined). Statistical analysis was carried out using the χ^2 test. OR: Odds ratio; 95% CI: 95% confidence interval.

TLR2 gene			Patients		Controls			Patients		Controls		
SNP	Nucleotide change M -> m[a]	Amino acid change	No.	Major allele frequency (number)	No.	Major allele frequency (number)	*P*-value	Genotype (number)	Genotype frequency	Genotype (number)	Genotype frequency	*P*-value
Novel	T 291->C	S97S	1	0.995 (193/194)	0	1 (204/204)	0.31	TT (96) TC (1) CC (0)	TT (0.99) TC (0.01) CC (0)	TT (102)	TT (1)	
Novel	C 413->T	T138I	1	0.995 (193/194)	2	0.99 (202/204)	0.57	CC (96) CT (1) TT (0)	CC (0.99) CT (0.01) TT (0)	CC (100) CT (2) TT (0)	CC (0.98) CT (0.02) TT (0)	
rs3804099	T 597->C	N199N	63	0.649 (126/194)	64	0.618 (126/204)	0.9	TT (34) TC (47) CC (16)	TT (0.35) TC (0.48) CC (0.17)	TT (38) TC (50) CC (14)	TT (0.37) TC (0.49) CC (0.14)	0.85
rs5743698	G 639->C	L213L	1	0.995 (193/194)	0	1 (204/204)	0.31	GG (96) GC (1) CC (0)	GG (0.99) GC (0.01) CC (0)	GG (102)	GG (1)	
Novel	G 798->C	L266F	1	0.995 (193/194)	0	1 (204/204)	0.31	GG (96) GC (1) CC (0)	GG (0.99) GC (0.01) CC (0)	GG (102)	GG (1)	
rs3804100	T 1350->C	S450S	16	0.91 (179/196)	16	0.923 (194/210)	0.83	TT (81) TC (15) CC (1)	TT (0.83) TC (0.16) CC (0.01)	TT (89) TC (16) CC (0)	TT (0.85) TC (0.15) CC (0)	0.58
rs5743700	C 1623->T	F541F	1	0.995 (195/196)	1	0.995 (209/210)	0.99	CC (97) CT (1) TT (0)	CC (0.99) CT (0.01) TT (0)	CC (104) CT (1) TT (0)	CC (0.99) CT (0.01) TT (0)	
rs5743704	C 1892->A	P631H	10	0.95 (196/206)	4	0.98 (206/210)	0.09	CC (93) CA (10) AA (0)	CC (0.90) CA (0.10) AA (0)	CC (101) CA (4) AA (0)	CC (0.96) CA (0.04) AA (0)	0.09
rs5743708	G 2258->A	R753Q	1	0.99 (205/206)	1	0.995 (209/210)	0.56	GG(102) GA (1) AA (0)	GG (0.99) GA (0.01) AA (0)	GG (104) GA (1) AA (0)	GG (0.99) GA (0.01) AA (0)	
rs5743709	G 2343->C	A781A	0	1 (206/206)	1	0.995 (209/210)	0.32	GG (103)	GG (1)	GG (104) GC (1) CC (0)	GG (0.99) GC (0.01) CC (0)	

[a]M = major, common allele in controls; m = less common allele in controls.

Table 3 Association analysis of TLR2 SNP obtained by allele discrimination real-time (Taqman) PCR of genomic DNA. Allele and genotype frequencies were obtained by dual allele-specific Taqman-based real-time PCR assays using DNA from indicated number of subjects of the case–control group. Statistical analysis was carried out as described in the legend to Table 1. See Materials & methods for explanation of Bonferroni correction of *P*-values, as we did not correct for multiple comparisons (nevertheless, a conservative Bonferroni adjustment would be multiplying our *P*-values by 2, which would still yield $P < 0.05$ values).

SNP		Major allele frequency[a,b]					Genotype frequency[a]					
M -> m[2]	aa	Patients (P)	Controls-1	Contact Controls-2	P versus C-1 *P*-value	P versus C-2 *P*-value		Patients (P)	Controls-1	Contact Controls-2	P versus C-1 *P*-value	P versus C-2 *P*-value
T 597->C	N199N	0.568 (216/380)	0.575 (466/810)	0.607 (102/168)	0.82	0.4	TT	0.347 (66/190)	0.328 (133/405)	0.345 (29/84)	0.65	0.97
							TC	0.442 (84/190)	0.494 (200/405)	0.524 (44/84)	0.24	0.21
							CC	0.211 (40/190)	0.178 (72/405)	0.13 (11/84)	0.34	0.19
T 1350->C	S450S	0.925 (344/372)	0.937 (875/934)	0.94 (158/168)	0.43	0.51	TT	0.855 (159/186)	0.876 (409/467)	0.881 (74/84)	0.47	0.56
							TC	0.14 (26/186)	0.122 (57/467)	0.119 (10/84)	0.54	0.64
							CC	0.005 (1/186)	0.002 (1/467)	0	0.48	
C 1892->A	P631H	0.936 (455/486)	0.956 (987/1032)	0.982 (167/170)	0.09	0.019[c]	CC	0.872 (212/243)	0.913 (471/516)	0.965 (82/85)	0.08	0.016[d]
							CA	0.128 (31/243)	0.087 (45/516)	0.035 (3/85)	0.08	0.016[e]
							AA	0	0	0		

[a]frequency (number / total number).
[b]M = major, common allele in controls; m = less common allele in controls.
[c]OR (95% CI): 3.79 (1.16–19.61).
[d]OR (95% CI): 0.25 (0.05–0.84).
[e]OR (95% CI): 4.0 (1.19–20.92).

Table 4. TLR2 haplotype and genotype frequency predictions by PHASE analysis. TLR2 SNP typing analysis from Table 3 was used to obtain Phase program predictions and statistical analysis for haplotype differences between patients and controls. Genotypes were estimated on the basis of best-pair predictions by the Phase algorithm. Statistical analyses were carried out using χ^2 test. OR: Odds ratio; 95% CI: 95% confidence interval.

Predicted haplotype frequencies

597–1350–1892	Total population		Controls		Patients*	
Haplotypes	E(freq)	S.E	E [Freq(0)]	S.E.(0)	E [Freq(1)]	S.E.(1)
TTC	0.52467	0.00617	0.53136	0.00729	0.51066	0.01104
TTA	0.05012	0.00060	0.04367	0.00038	0.06362	0.00153
TCC	0.00413	0.00153	0.00408	0.00178	0.00424	0.00210
CTC	0.35946	0.00630	0.36204	0.00739	0.35408	0.01119
CTA[a]	0.00003	0.00014	0.00001	0.00010	0.00006	0.00038
CCC	0.06158	0.00286	0.05884	0.00289	0.06732	0.00588
CCA[a]	0.00001	0.00012	0.00001	0.00010	0.00002	0.00020

[a]Haplotypes CTA and CCA were not found in the population.
*P = 0.63 (cases versus controls).

Genotype best-pair estimates

	Controls	Patients	Controls versus Patients	Contact Controls	Contact Controls versus Pt
Genotypes	*n*	*n*	p	*n*	p
1.1	111	55		26	0.12
1.2	35	23	0.096	2	0.036[a,b]
1.3	2	1		1	
1.4	250	107		38	0.82
1.5	37	13		6	0.54
2.4	8	6	0.4	1	0.49
2.5	2	2	0.4	0	
4.4	55	29		8	0.56
4.5	16	10		3	0.56
5.5	1	1		0	
	517	247		85	

[a]OR (95% CI): 4.26 (1.01–37.97).
[b]all genotypes containing haplotype 2: P = 0.018, OR (95% CI): 3.92 (1.17–20.53).

expression vector pLNOH2 (Fermentas Life Sciences, Ontario, Canada). The TLR2-P631H positive pLNOH2 recombinant plasmids (as determined by the allele-specific real-time PCR) were sequenced using the primers described in Table 1. Recombinant plasmid with the correct orientation of inserts, mutation and nucleotide sequence was designated pLNOH2-TLR2-P631H, and used to make the final construct for transfection studies (we found that this construct had an additional inhibitory effect in our transfection studies compared to the TLR2 construct which was embedded in the pCDNA3.1 plasmid, so we aimed to avoid it). The final construct used for transfection studies presented in this work was generated by PCR as follows: the TLR2 gene fragment (2359 bp) was amplified from the pLNOH2-TLR2-P631H recombinant plasmid with the primer pair TLR2-FL-F and TLR-FL-R (Table 1) using the PfuUltra high-fidelity DNA polymerase (Stratagene, CA, USA) and cloned into the pCDNA3.1 plasmid (Directional TOPO Expression system; Invitrogen, CA, USA) according to manufacturer's protocols. The cloned fragment was checked by DNA sequencing with the primers described in Table 1. Recombinant plasmid with the correct sequence harbouring the TLR2-P631H mutation was designated pCDNA3.1D-TLR2-P631H. The wild type TLR2 allele used for transfection studies was reported previously [23].

Cell signalling studies. Cotransfections: HEK293 cells were transfected or cotransfected with various amounts of plasmid DNA constructs containing wild type TLR2 and TLR2-P631H alleles (up to 100 ng each). After 5–6 h of stimulation with 50 ng/ml Pam3CysSK4, 5 μg/ml LTA, 10 ng/ml lipomannan (LM), 1 μg/ml liporabinomannan (LAM) or 10 μg/ml FSL-1, cells were lysed and NF-κB luciferase reporter gene activity was measured. IL-1ß or TNF (100 ng/ml) was used as a positive control. Signalling activity was expressed in relative luminescence units of NF-κB-dependent firefly luciferase activity. Cells were also transfected with pRL-TK *Renilla* luciferase control plasmid (Promega) as a control for transfection efficiency

and cell viability. The dual luciferase assay system (Promega) was used to measure ELAM-Luc and *Renilla* luciferase activity. The latter was used to normalize data as described in further detail in Fig. 3 legend. Data shown are the mean ± SD of triplicate wells.

Ligands: Synthetic tripalmitoyl cysteinyl lipopeptide (Pam3CysSK4) and fibroblast-stimulating lipopeptide-1 (FSL-1) were purchased from EMC Microcollections (Tübingen, Germany). LTA isolated from *S. aureus* [24] was a kind gift from Dr Sonja von Aulock (University of Konstanz, Gremany). LM and LAM from *M. smegmatis* were purchased from Invivogen (CA, USA). Heat-killed *Mycobacterium avium* bacteria were a kind gift from Dr Trude Flo (Norwegian University of Science and Technology, Trondheim, Norway). Recombinant human IL-1ß and TNFα were purchased from R&D Systems.

Cell internalization studies. Antibodies: Anti-TLR2 mouse monoclonal antibodies TL2.1 and TL2.3 were reported previously [23]. Secondary anti-mouse antibodies (conjugated either to Alexa 488 or Alexa 546) were from Molecular Probes. Alexa-conjugated primary antibody was generated by protein coupling to Alexa 555 (Invitrogen) according to the manufacturer's instructions.

Cell growth: Regular Madine Darby Canine Kidney (MDCK) cells and HEK-G cells stably expressing early endosomal antigen 1(EEA1)-green fluorescent protein(GFP) were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 9% foetal calf serum (FCS), 2 mM glutamine, 25 U/ml penicillin and 25 μg/ml streptomycin at 37 °C, with air supply containing 6% CO_2.

Transfection techniques: Regular Madine Darby Canine Kidney and HEK-G cells were seeded overnight on either glass coverslips or Microwell dishes for microscopy (Mattek, MA, USA). Cells were transiently transfected with plasmids (pCDNA3) containing the wild type TLR2 or TLR2-P631H alleles using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were incubated at least 16 h prior to internalization experiments.

Immunostaining and internalization experiments: For regular immunostaining, cells grown on glass coverslips and transfected with TLR2 constructs were fixed with paraformaldehyde (PFA) or methanol prior to staining with TL2.1 or TL2.3 antibodies and appropriate secondary antibodies. For regular internalization experiments, cells grown on glass coverslips and transfected with TLR2 constructs were labelled with TL2.1 or TL2.3 antibodies on ice for 30 min, and incubated in prewarmed growth medium at 37 °C in the incubator for various amounts of time to let the surface-labelled antibodies internalize. The cells were then fixed with PFA followed by incubation with 0.1% saponin and labelled with the appropriate secondary antibody. Images were recorded on Olympus Fluoview F1000 with a 100× oil objective. For live imaging experiments, cells were supplemented microscopy medium, DMEM without phenol red and sodium carbonate, supplemented with 3.5 g/l D-glucose to a final concentration of 4.5 g/l; 25 mM HEPES; with 10% FCS. Cells were kept on ice for 45 min and then incubated with conjugated antibody on ice for an additional 45 min. Live image acquisition was performed on an Andor Revolution XD spinning disc microscope. Microscope platform was the Olympus IX 71 with PlanApo N 60x/1.42 NA oil immersion objective. The spinning disc unit CSU22 was synchronized with an iXon^{EM+} 885 EMCCD camera. A stable cellular environment was provided by a 37 °C microscope chamber delivered by Solent Scientific. Internalization analysis was performed on the Andor iQ multidimensional image software quantifying average fluorescence. The data obtained from the iQ software were normalized and corrected against the fluorescent background and the relative values plotted as a function of time.

Results

Sequencing DNA of TLR2 alleles

We sequenced coding regions of TLR2 alleles in 103 patients with tuberculosis and 105 healthy persons in Croatian population by sequential amplification of genomic DNA using high-fidelity genomic PCR comprising five sets of primers described in Table 1. The DNA sequences of both strands were compared to the reference sequence and differences are listed in Table 2. We found in total 10 SNP, and all but three were already reported in the Ensemble SNP reference database. Of the three novel SNP (Table 2), two were non-synonymous: T138I and L266F, and the third one was synonymous: S97S (Fig. 1). The latter two were single occurrences, and the former was found in three individuals. Only four single nucleotide mutations produced different amino acids than those listed in the reference sequence (T138I, L266F, P631H and R753Q). The remaining six SNP were synonymous amino acid exchanges (S97S, N199N, L213L, S450S, F541F, and A781A). We found no deletions or insertions in sequenced samples. Several mutations were associated with others: N199N was found with one of the four mutations S97S, T138I, L213L and L266F in four patients, and also with T138I in two control subjects, but we were unsure whether they represented single haplotypes (Table 2).

The most prominent difference between patients and controls, a mutation producing a proline into histidine change at aa position 631 (P631H), was found in ten patients and in four control subjects (Table 2) showing a trend for significant difference ($P = 0.09$) when compared. The other interesting mutation (found in two persons) was the G2258A, which changed an amino acid at

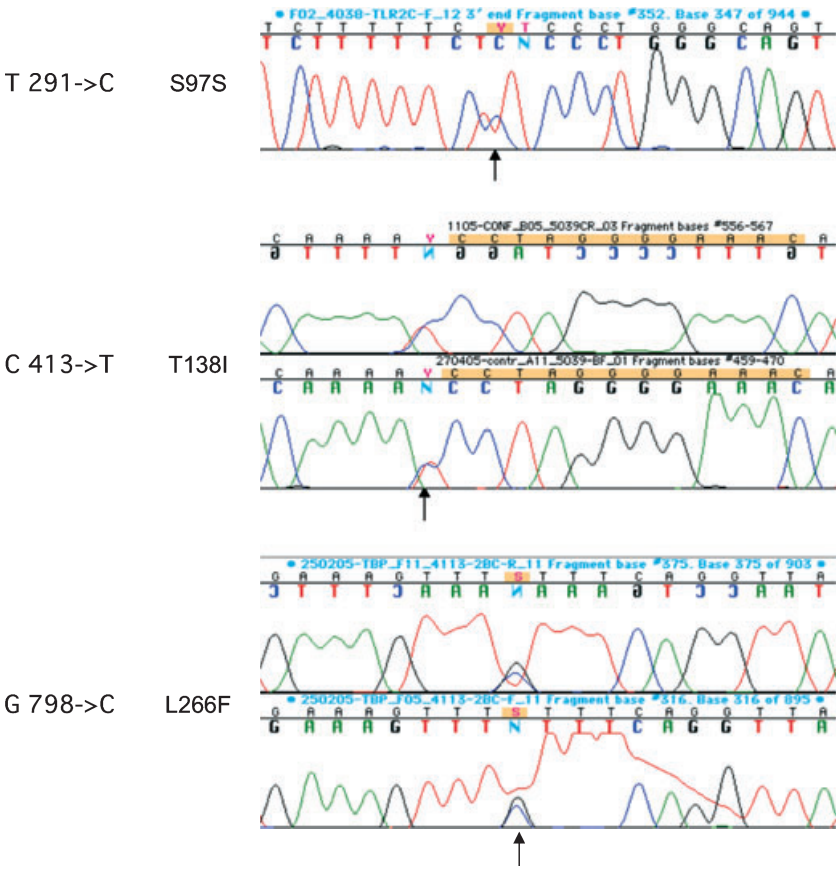

Figure 1 Novel TLR2 single nucleotide polymorphisms found by sequencing DNA of Croatian Caucasian population. DNA sequencing was performed as described in *M&M*.

position 753 (R753Q) (Table 2). Both changes affect the intracellular portion of the molecule and were thus possible candidates for aberrant signalling. On the other hand, most of the observed extracellular mutations yielded no changes in the amino acid sequence except the two novel ones: T138I, and the L266F (Table 2).

SNP analyses by allele discrimination PCR and haplotype/genotype predictions

We next used Taqman-based allele discrimination PCR method to check the allelic and genotypic frequencies of the three most frequent alleles (N199N, S450S and P631H) in the rest of our case–control group. The results are presented in Table 3. The comparison indicated a significant difference between the number of genotypes containing TLR2-P631H allele between the patients (n = 247) and contact controls (P < 0.02; 95% CI 1.17–20.92; n = 85). However, these differences became less pronounced when compared to general population (healthy blood donors; n = 517) as they showed only a trend towards significance (P = 0.09). Not all patients and controls were typed for each of these SNP. Those with missing alleles were subsequently used for haplotype/genotype prediction by the Phase software.

By genotyping the three most frequent SNP in our case–control group, we obtained only the allelic content of each locus. From these unknown gametic phases (Table 3), the Phase program predicted the most likely haplotypes and genotypes for each individual, and their population frequencies are shown in Table 4. The most frequent predicted haplotype containing the P631H mutant in our case–control group was TTA (representing sequentially alleles at positions 597, 1350 and 1892). The comparison between patients and healthy controls showed a trend towards significance for subjects having TTA in combination with TTC haplotype. This genotype was significantly associated with disease in comparison between patients and contact controls (P = 0.036, 95%CI: 1.01–37.97).

Intracellular signalling studies with transfected TLR2-P631H allele

To ascertain whether the association with disease could be because of a difference in function of the mutant allele from the wild type TLR2 allele, we studied the expression and signalling *in vitro* of the TLR2-P631H variant by transient transfection into human embryonic kidney (HEK293) cells. These cells express TLR1 and TLR6, but lack the expression of TLR2 (data not shown). Upon

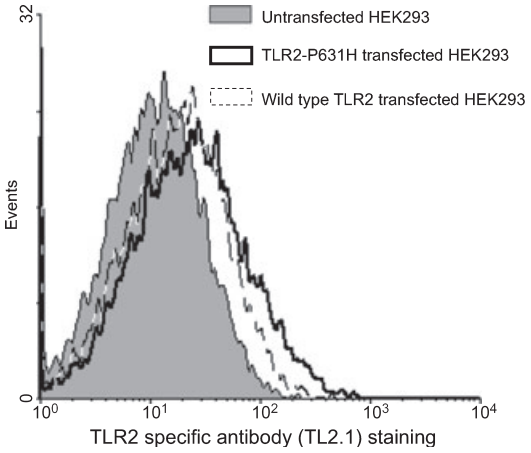

Figure 2 TLR2-P631H mutant is expressed on the cell surface of transfected HEK293 cells. Both constructs TLR2-P631H mutant and wild type TLR2 were transfected in HEK293 cells by transient procedure and labelled with TLR2-specific antibody TL2.1, as described in *M&M*.

transfection, the expression of the mutant TLR2-P631H allele on the cell surface of transfected HEK293 cells was determined by staining with the anti-TLR2 mAb TL2.1. There were no differences in the level of expression between the wild type and mutant alleles (Fig. 2).

To determine the influence on NF-κB signalling, we transiently cotransfected the mutant TLR2-P631H construct together with the NF-κB-driven luciferase as a reporter gene. We then stimulated the transfected cells with a range of TLR2 ligands including Pam3CSK4 (that binds the TLR2/TLR1 heterodimer) [11, 25] and FSL-1 (known to bind the TLR2/TLR6 heterodimer) [6, 26]. Transient transfection of the mutant gene construct diminishes the NF-κB signal transduction initiated by both the Pam3CSK4 ligand and the FSL1 (as ascertained by measuring the level of luciferase luminosity) from the levels seen with the wild type TLR2 construct (Fig. 3A). Other TLR2 ligands like LM, LAM (data not shown), LTA or heat-killed *Mycobacterium avium* extracts similarly inhibited NF-κB signal transduction (Fig. 3A).

In double-allele transient cotransfections, using various amounts of the wild type and mutant constructs, the P631H allele was unable to activate NF-κB to the same extent as the wild type TLR2, when stimulated either with the Pam3CSK4 or the FSL-1 ligand, indicating a dominant negative effect (Fig. 3B). However, this inhibition was a mild one, because the NF-κB activity was at approximately 80% of that of the wild type receptor alone, when equal amounts of cotransfected alleles were used (Fig. 3B), and was observed at the greatest extent (45% inhibition) with the LM ligand (Fig. 3C). Increased amounts of the mutant allele in cotransfections with the wild type receptor allele further lowered the reporter gene activity producing about 50% inhibition for the Pam3CSK4 or the FSL-1 ligands (Fig. 3B) and over 65% impairment for the LM ligand (Fig. 3C). This dose-dependent inhibition of signalling was also seen with *M. avium* extracts as shown in the Fig. 3D, indicating a possibility for weakened recognition of related *M. tuberculosis*.

There appeared to be a discrepancy between the mild dominant negative effect observed *in vitro*, and the finding that the P631H mutation was never found in homozygosity, which might be an indication of a more deleterious effect of the mutant *in vivo*. It is possible that, at physiological conditions, the mutant might have altered the balance with the wild type at the cell surface in its favour, thereby amplifying the dominant negative effect. One possibility to achieve this would be to affect the rate of its internalization, and for this reason we chose to study it.

Cell internalization studies

Upon ligation, specific anti-TLR2 antibodies were specifically taken up by the cells expressing TLR2 and could be tracked down to the regular endosomal/lysosomal pathway. MDCK cells stably expressing the early endosomal antigen fused to GFP (EEA1-GFP) were cotransfected with the wild type TLR2 and TLR2-P631H allele and incubated with the TLR2.1 antibody conjugated to Alexa 555. Supplementary videos 1 and 2 and Fig. 4B show the uptake of the fluorescently labelled TLR2.1. According to this data, the wild type TLR2 and TLR2-P631H were expressed on the cell surface of cotransfected cells at approximately equal amounts and were internalized. The mutant seemed to internalize at a lower rate than the wild type TLR2 (Fig. 4A) and progress more slowly towards late endosomes/lysosomes (data not shown). In cells transfected with the wild type TLR2 allele, we could detect to a larger extent the antibody colocalizing with early endocytic compartments compared to the mutant (Fig. 4B white arrows). Similar results were obtained when we used MDCK or HEK-G cells transiently transfected with the wild type or the mutant TLR2 that were labelled with TLR2.1 and TLR2.3 antibodies and fixed at various time intervals (data not shown).

We quantified the internalization of transiently transfected TLR2 wild type and TLR2-P631H in MDCK cells also expressing the EEA-GFP construct. Figure 4A shows the kinetics of internalization of TLR2.1 antibody in these cells as a function of time, and Fig. 4B shows cell surface and internal labelling of TLR2.1 antibody (red) and EEA1-GFP (green) at representative time points. It should be noted that TLR2.1 antibody was present in the medium throughout the entire experiment. Only around 20% of the surface-labelled TLR2.1 was internalized during the 40-minute span in cells transfected with TLR2 P631H (and the plateau was reached after 20 min),

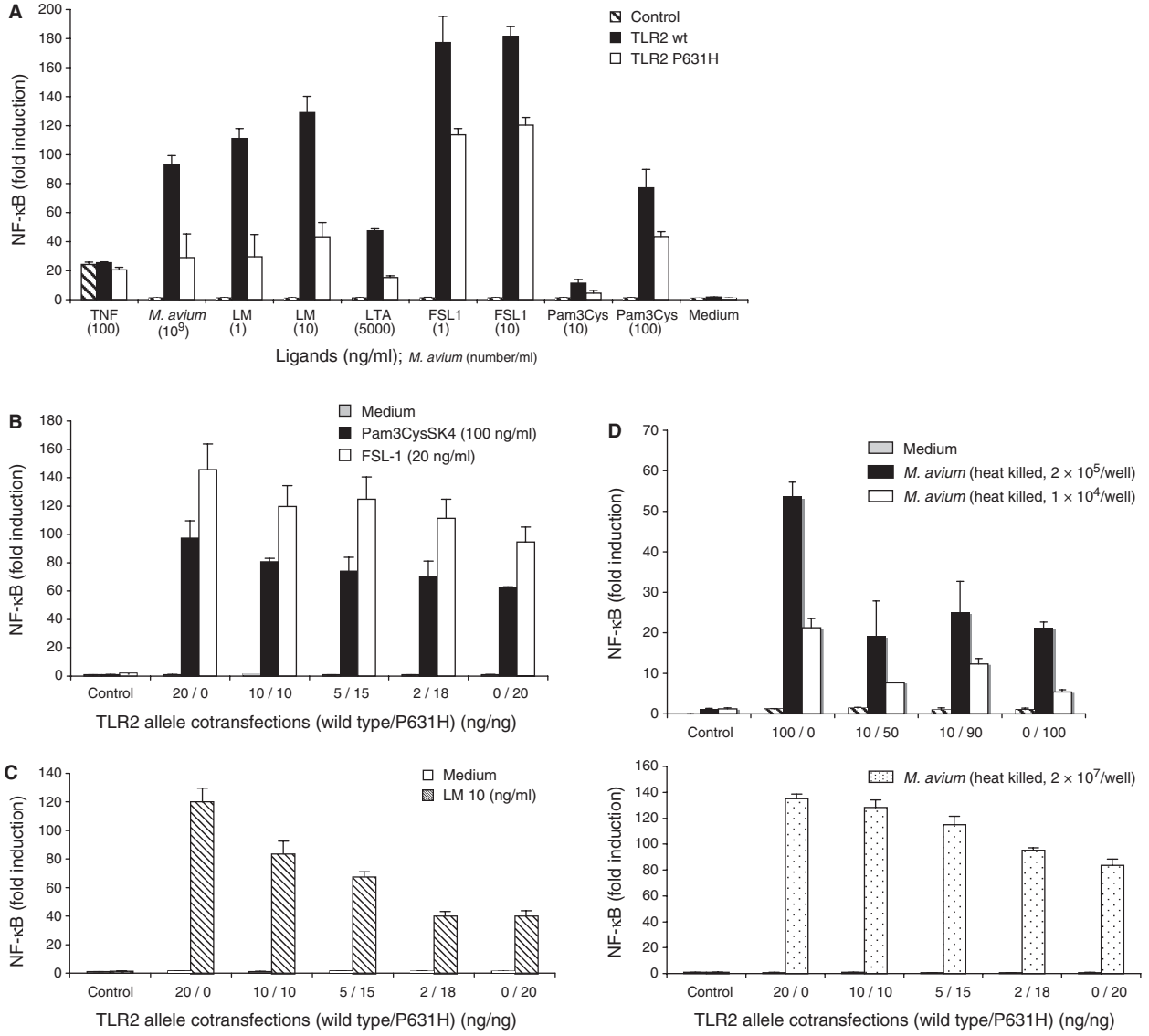

Figure 3 TLR2-P631H mutant inhibits NF-κB activation in HEK293 cells in stimulation with TLR2 ligands in a dominant negative fashion. (A) The responses of HEK293 cells to stimuli by various ligands were inhibited by TLR2-P631H. Cells were transfected either by wild type TLR2 allele or TLR2-P631H. After 6 h of stimulation with ligands, cells were lysed and NF-κB luciferase reporter gene activity was measured. As controls, the *Renilla* luciferase reporter system was also cotransfected. TNF-α stimulation was used as a positive control for the transfection of the ELAM/NF-κB reporter gene construct. Data are shown as the mean ± SD of triplicate wells. (B) The dominant negative response of HEK293 cells to stimuli by Pam3CysSK4 and FSL-1 ligands in cotransfection experiment with double alleles. Cells were transfected either by the wild type TLR2, TLR2-P631H or by the indicated ratio of mutant to wild type alleles (and with *Renilla* luciferase). The cells were cotransfected, assayed and presented similarly as described in 3A, except that two normalizations were done: one with the TNF-α levels (to check for internal NF-κB levels) and to *Renilla* luciferase luminosity (to check for transfection efficiencies). (C) The NF-κB fold increase by stimulation with lipomannan (LM) is progressively diminished by the TLR2-P631H construct in a dominant negative fashion upon cotransfection with the wild type allele in HEK293 cells. The cells were cotransfected, assayed and presented (with normalizations) as described in 3B. (D) The responses of HEK293 cells to stimuli by heat killed *M. avium* were progressively diminished by the TLR2-P631H construct in a dominant negative fashion. The cells were transfected either by the wild type TLR2, TLR2-P631H or by the indicated amounts of mutant and wild type alleles. The cells were cotransfected in two separate experiments (upper and lower graphs), stimulated by two or single concentrations of *M. avium* extracts, and presented similarly as described in 3B. However, the upper graph represents raw data, and the TNFα and *Renilla* luciferase normalizations were done for the experiment shown at the bottom.

whereas almost 60% was internalized in cells transfected with the wild type TLR2 and at some stage the internalized fraction of antibody colocalized with the early endosomal marker EEA1 (Fig. 4B). Furthermore, when we labelled MDCK or HEK-G cells transfected with either the wild type or the mutant TLR2 with TLR2.3 antibody, allowed it to internalize for up to 60 min and fixed the cells, we saw that the TLR2-P631H

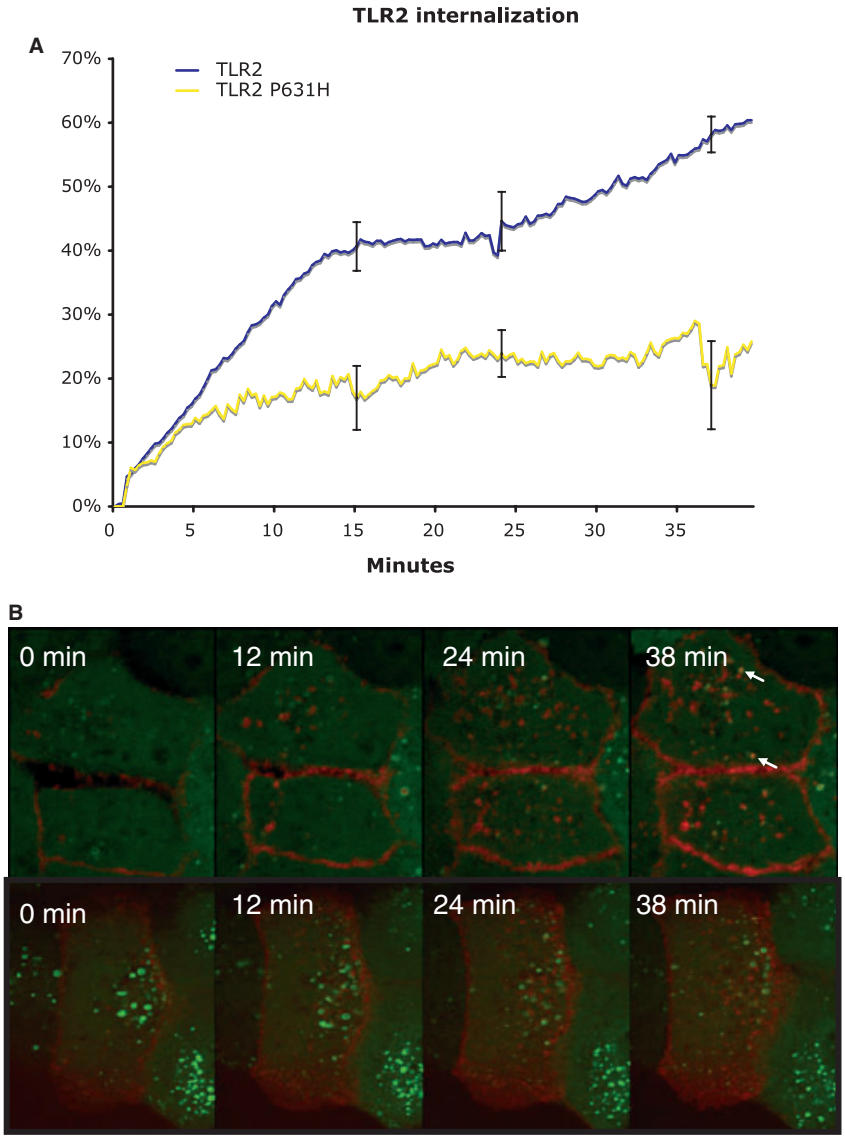

Figure 4 TLR2-P631H affects rate of internalization of the wild type TLR2. MDCK cells stably transfected with EEA1-GFP were transiently transfected with either the wild type TLR2 or TLR2-P631H DNA for 24 h prior to the experiment. Cells were then supplemented with microscopy medium, DMEM without phenol red and sodium carbonate, containing 4.5 g/l D-glucose, 25 mM HEPES, and 10% FCS. Cells were kept on ice for 45 min and then incubated with fluorescence conjugated antibody on ice for additional 45 min prior to switching to the 37C Andor Spinning Disk microscope chamber. (A) Plot of relative internalized TLR2 wild type and TLR2-P631H as a function of time. Mean of five independent experiments are plotted, standard deviations were calculated continuously, and standard deviations at representative time points are plotted. (B) Confocal images at representative time points: 0, 12, 24 and 38 min after shifting of the cells to 37C. Top row: cells transfected with wild type TLR2; bottom row: cells transfected with TLR2-P631H. Red, Alexa 555 – conjugated TLR2.1, green, EEA1-GFP construct.

internalized at least 50% less than the wild type TLR2 (data not shown). We therefore concluded that TLR2-P631H internalization was significantly lower than that of the wild type TLR2 at similar surface expression levels.

Discussion

The TLR2-P631H mutant allele is significantly overrepresented in tuberculosis when patients were compared to contact controls, indicating a possible low-risk predisposition. However, TLR2-P631H showed only a trend towards significance in comparison of diseased with general Caucasian (Croatian) population. The difference between the general population and the contact controls is that the latter should be devoid of the 5–10% of the general population that can fall ill with tuberculosis. Our result fits with a notion that genetic susceptibility to tuberculosis is of complex nature including several unknown yet distinctive loci each of which contribute to a small extent and at different rates in various human populations. So far, numerous case–control studies reported associations with tuberculosis for genes with roles either in innate or adaptive immunity [12–14, 27–35]. Other molecules like Vitamin D receptor [36, 37], purinergic receptor P2X [38], and 'Natural resistance–associated macrophage protein-1' (NRAMP1) gene [33–35] were also found associated with disease. However, in different populations, some of these genes affected only disease severity. Similarly controversial is the involvement of the genes of the IFN-γ/interleukin-12 (IL-12) axis [18–20, 39–44].

Single nucleotide polymorphisms within the TLR2 gene (R677W; and R753Q) were found to correlate with susceptibility to tuberculosis in Turkish and Tunisian

populations, respectively [12, 13]. However, these reports relied on too few patients, and were thus amendable to statistical bias. Furthermore, the association was found with meningeal rather than pulmonary tuberculosis with the TLR2-T597C allele in Vietnamese population, and the study suggested that TLR2 mutations could affect dissemination of *M. tuberculosis* within the body [17]. As the T597C is a synonymous mutation (N199N), it is likely that it is only a marker for another predisposing allele. We found no correlation with the disease when TLR2-T597C allelic/genotypic frequencies were analysed alone (Table 3), but our cases lacked meningeal tuberculosis.

The reason why none of the previously reported TLR2 SNP/disease associations were found in our case–control study with Caucasians of Croatian origin is most likely because of the rare occurrence or absence of these SNP in Caucasians. For example, in our samples, of 416 sequenced TLR2 genes we found only two R753Q SNPs, and none of the R677W kind. The latter SNP seemed to be absent from Caucasian population, as another study reported only R579H, P631H and R753Q alleles present [45]. Therefore, in Croatian Caucasians, the P631H variant might take the role in the association with susceptibility to tuberculosis that those previous studies from Turkey and Tunis claimed for other TLR2 mutations.

We next wished to assess whether the TLR2-P631H mutant affects intracellular signalling. It is known that TLR2 homodimers were not sufficient to initiate signalling [26], and complementation experiments with chimeric proteins implied that multimerizations of either TLR2 with TLR1 or TLR6 are required for cells to recognize various ligands [25]. TLR1 and TLR2 signalling is important for detecting triacylated lipopeptides and various bacterial products [46] including *B. burgdorferi* outer-surface lipoprotein [47] and mycobacterial lipoprotein [6]. A functional interaction between TLR2 and TLR6 is necessary for responses to group B streptococci [48], diacylated lipopeptide MALP-2 isolated from *Mycoplasma fermentans* [6], and phenol-soluble modulin [49]. Here we showed that expression of the TLR2-P631H mutant alone (and likely in association with the HEK293 cells' endogenous TLR1 and TLR6) impaired the response of HEK293 cells to TLR2 ligands Pam3CSK4, FSL-1, LTA, LM, and heat killed *Mycobacterium avium*. Furthermore, this is the first report that a dominant negative (suppressive) action of the TLR2-P631H can be exerted on the wild type allele upon double-allele cotransfection of HEK293 cells, when stimulated by ligands that bind both forms of TLR2 heterodimers: the TLR1/TLR2 (Pam3CSK4, and *M. avium* extract) and the TLR2/TLR6 (FSL-1).

A mouse homologue Tlr2-P681H has a similar dominant negative action on TNF-α secretion in macrophages using the *M. tuberculosis* lipids as ligands [50]. Both human and murine TLR2/Tlr2 signal via MyD88 molecule, which binds the receptor intracellularly. According to Brown *et al.* (2006) [51], the murine Tlr2-P681H mutation does not affect the overall signalling domain fold predicted by the TIR crystal structure, but might instead destroy MyD88-dependent signalling observed by Xu *et al.* (2000) by determining the topology of the critical fold and disturbing a critical spatial arrangement of contact residues [52]. Brown *et al.* further corroborate this statement by showing that the murine P681H mutation did not completely destroy TLR2 - MyD88 interactions in their yeast 2-hybrid assays, unlike the Tlr2-P681H that entirely destroyed them [51].

We then studied internalization of TLR2 variants. Although intracellular distribution of the TLR2-P631H mutant was very similar to that of the wild type, the internalization of the mutant from the plasma membrane was significantly slower (Fig. 4A). In addition, it seems that a greater part of internalized structures (anti-TLR2 antibody together with the TLR2 molecule) utilizes other than well-described early endosomal compartments (by EEA-1) to gain access to cellular interior. This was found in both, the wild type and the mutant allele, with a slight increase in the case of the latter (data not shown). In conclusion, this is the first evidence that TLR2-P631H inhibits internalization of the TLR2 complex, the consequence of which needs to be studied further. In addition, a separate study should be designed in the future to address a potential internalization slow-down caused by a similar mutation in the mouse.

The relevance of the TLR2 cell trafficking on its function is not clear. A recent study on internalization indicates that it was not required for successful TLR2 intracellular signalling [53]. However, it remained possible that difference in the rate of internalization might somehow affect the extent of signalling. As a consequence, TLR2-P631H might simply depress the unchanged cytokine release profile in responding cells. Alternatively, the TLR2-P631H could produce different outcome of the immune response, perhaps in a similar way as the TLR2-R753Q causes altered cytokine release in response to *Candida* (but not to Gram-positive sepsis) [54]. Blocking internalization of the wild type TLR2 by immobilizing the ligand (LTA) resulted in augmented response of cells [53]. Perhaps, the increased levels of the immobilized ligand-receptor complexes at the cell surface were able to recruit a larger amount of signalling molecules, thereby enhancing the cell's response. Would the opposite have happened, where the dominant negative variant of the TLR2 become similarly stuck at the cell membrane? We hypothesize that the slower internalization of TLR2-P631H might have induced a misbalance in the ratio of the TLR2 variants expressed under physiological conditions (i.e. in cells heterozygous for the mutant), leaving more mutant than wild type molecules

at the cell surface (Fig. 4A,B). This could in turn intensify the otherwise mild dominant negative effect similar to what we observed in our double-allele transfection experiments using increasing amounts of the mutant receptor (Fig. 3B–D). We believe that the TLR2-P631H might affect at least the extent if not the profile of the cellular response upon binding the ligand. Therefore, a different cytokine profile during infection, as a result of this particular TLR2 mutation, might modify severity or outcome of disease.

As our evidence suggests that the extent of suppression of intracellular signalling by the TLR2-P631H mutant was surprisingly mild, it is conceivable that in certain conditions this might lead to beneficial consequences. It could prevent a septic shock with a devastating cytokine burst in some infectious diseases like, for example, meningitis. Responding without the damaging septic shock during infectious diseases (or having a milder form of it) would be of selective advantage, in general. Indeed, we might find a support for this in a report showing that the TLR2-P631H mutant was underrepresented in meningococcal meningitis [55] perhaps assuming a protective role. We suggest that a putative protection against meningitis also carries a slightly increased risk for tuberculosis, thus enlarging the complexity of genetic predisposition to the latter.

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