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CUTTING EDGE

Cutting Edge: A Common Polymorphism Impairs Cell Surface Trafficking and Functional Responses of TLR1 but Protects against Leprosy[1]

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TLRs constitute an essential family of pattern recognition molecules that, through direct recognition of conserved microbial components, initiate inflammatory responses following infection. In this role, TLR1 enables host responses to a variety of bacteria, including pathogenic species of mycobacteria. In this study, we report that I602S, a common single nucleotide polymorphism within TLR1, is associated with aberrant trafficking of the receptor to the cell surface and diminished responses of blood monocytes to bacterial agonists. When expressed in heterologous systems, the TLR1 602S variant, but not the TLR1 602I variant, exhibits the expected deficiencies in trafficking and responsiveness. Among white Europeans, the 602S allele represents the most common single nucleotide polymorphism affecting TLR function identified to date. Surprisingly, the 602S allele is associated with a decreased incidence of leprosy, suggesting that* Mycobacterium leprae *subverts the TLR system as a mechanism of immune evasion. *The Journal of Immunology,* **2007, 178: 7520–7524.**

As pattern recognition molecules, TLRs constitute critical components of the innate immune system that function to sense the presence of pathogens and initiate inflammatory responses (1, 2). In addition to this important role, TLRs provide an essential bridge between innate and adaptive immunity (3). Humans possess 10 TLR family member subsets, which are expressed in a variety of tissues and cell types throughout the body. Individual TLR family members exhibit exquisite specificity for bacterial, fungal, and viral components and initiate immune responses appropriate to the class of invading pathogen.

TLRs 1, 2, and 6 are a phylogenetically related subfamily. High sequence similarity and tandem arrangement of TLRs 1 and 6 in the human genome suggest that these two receptors arose from a recent gene duplication event (4, 5). TLR2 requires either TLR1 or TLR6 as a coreceptor, which confers ligand specificity and enables cell signaling (6). Collectively, these receptor pairs mediate immune responses to a wide variety of acylated cell wall components derived from Gram-positive bacteria, Gram-negative bacteria, mycoplasma, spirochetes, and fungi (1, 2). In accordance with their broad recognition capability, mice deficient in TLR2 exhibit increased susceptibility in a variety of acute infection models (7). Clinical studies have revealed associations between infectious disease and single nucleotide polymorphisms (SNPs)[3] within TLR genes, highlighting the importance of these receptors in human immune defense (8, 9).

Mycobacterium leprae is the causative agent of leprosy, a chronic disease of the skin and peripheral nervous system in which an estimated 700,000 new cases are detected annually (10). The host response to *M. leprae* exposure is quite variable, and mounting evidence strongly suggests that a variety of host genetic factors play a role in determining the extent and severity of infection (11). *M. leprae* bacilli, as well as several mycobacterial cell wall components, are agonists for the TLR1/2 receptor pair, including lipomannan, lipoarabinomannan, phosphatidylinositol dimannoside, and a 19-kDa lipoprotein (12–15). TLRs 1 and 2 are expressed by a variety of cell types in both the dermis and epidermis of the skin, and their expression has been detected in leprosy lesions by immunostaining (15). In this study, we report that a frequently occurring SNP in *TLR1*, which impairs receptor trafficking and function, plays a protective role in the context of clinical leprosy, indicating that TLR1/2 signaling during *M. leprae* infection may be detrimental to the host.

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[3] Abbreviation used in this paper: SNP, single nucleotide polymorphism.

Materials and Methods

Reagents

Synthetic lipopeptides were purchased from EMC Microcollections. Repurified LPS (*Salmonella minnesota* Re595) was purchased from List Biological Laboratories. Heat-killed *Staphylococcus aureus* and *Acholeplasma laidlawii*, as well as *Porphymonas gingivalis* LPS, were obtained from InvivoGen.

Cells and cell culture

Human PBMCs were isolated from the blood of healthy donors by centrifugation through a Ficoll gradient. For experiments requiring a pure monocyte population, isolation was performed by negative selection over magnetic beads (Miltenyi Biotec). Monocyte preparations were of >95% purity as determined by staining for CD14.

Flow cytometric analysis

Cell surface expression of endogenous TLR1, TLR2, and TLR6 was measured using mAbs GD2.F4, T2.5, and hPER6, respectively (eBioscience). Appropriate isotype-matched Abs were used as controls. Ab staining was followed by biotinylated secondary Ab and PE-conjugated streptavidin (Jackson ImmunoResearch Laboratories). To detect intracellular levels of TLRs, cells were fixed in PBS containing 2% (w/v) paraformaldehyde and permeabilized with 0.1% (w/v) saponin before staining.

Cell stimulation

SW620 cell and 293T cell transfections, stimulation, and data analyses were performed as described previously (16). Human PBMCs were seeded in 96-well plates at a density of 1×10^5 cells/well and stimulated with the indicated agonists for 16 h. The concentration of secreted human TNF-α was measured in the supernatant using a paired Ab TNF-α sandwich ELISA (Invitrogen Life Technologies).

Genotyping of blood donors

A fragment containing exon 4 of *TLR1* was amplified from genomic DNA using the following PCR primers: forward, CTTGATCTTCACAGCAATA AAATAAAGAGCATTCC, and reverse, GGCCATGATACACTAGAACA CACATCACT. *Pst*I digestion of the product was used to discriminate between the *TLR1 602* alleles. Validity of this assay was confirmed by sequencing DNA from several donors of each genotype.

Immunoblotting

Whole lysate or immunoprecipitates from transfected 293T cells were separated on a 7.5% SDS-polyacrylamide gel followed by Western blotting using either anti-FLAG M2 Ab (Sigma-Aldrich) or anti-HA.11 Ab (Covance).

Confocal microscopy

Primary monocytes were plated in chamber slides (Labtek) and allowed to adhere overnight. Cells were then washed, and one group was fixed with 2% paraformaldehyde, followed by permeabilization with 0.5% Triton X-100. Cells from the other group were untreated. After blocking FcRs, Ab staining was conducted using anti-TLR1 and a Signal Amplification kit (Molecular Probes). Images were acquired on a Zeiss LSM 510 scanning confocal laser microscope.

Leprosy samples

Leprosy patients were recruited from two main centers located in endemic areas in Turkey: Leprosy Hospital, Elazig, and Akdeniz University, Antalya. Leprosy diagnosis was based on clinical findings and laboratory tests, which usually involved either a blood smear and/or skin biopsy. *M. leprae* was identified by the presence of acid-fast bacilli and histopathological changes. Differential diagnoses, including granulomatous disease, syphilis, tuberculosis, sarcoidosis, and deep fungal infection, were excluded by clinical and laboratory criteria. Asymptomatic control subjects were recruited from the same centers and had neither family history nor symptoms related to leprosy. Protocols involving human subjects were approved by human subject review committees at University of Illinois, Humboldt-University Berlin, and Akdeniz University. Written informed consent was obtained from all blood draw participants, control subjects, and leprosy patients.

Genotyping of leprosy samples

Genotyping was performed on genomic DNA using fluorescence-labeled hybridization as described previously (17). The PCR contained the primers TGTGACTACCCGGAAATTATAGA and CCCAGAAAGAATCGTGCC along with the hybridization-fluorescence resonance energy transfer probes CCATGCTGGTGTTGGCTGTGACTGTG-FL and LCRed640-CCTCC CTCTGCATCTACTTGGAT. The *TLR1 602I* and *602S* alleles gave rise to melting peaks at 64.5 and 58.3°C, respectively. The genotyping protocol was verified on several samples.

Statistical analysis

ANOVA and posthoc statistical analyses, including Fisher's exact test, were performed using algorithms provided in SPSS software version 14.0.

Results and Discussion

Homozygosity for TLR1 602S is associated with impaired cell surface expression and function

Upon staining human peripheral blood monocytes for TLRs using a sensitive biotin-streptavidin protocol, we observed that TLR2 and TLR6 were readily detected. However, monocytes obtained from some blood donors completely lacked detectable cell surface of levels of TLR1 (Fig. 1*A*). To determine whether this reflected a complete absence of TLR1 expression, we performed flow cytometric analysis on permeabilized monocytes and found no significant difference in total TLR1 protein levels between cells that expressed surface TLR1 and cells that did not (Fig. 1*A*). Taken together, these results demonstrate that some individuals lack surface expression of TLR1 without exhibiting any deficiency in total cellular levels of receptor.

Fluorescence microscopy revealed a punctuated pattern of TLR1 staining on monocytes derived from individuals who express surface TLR1 (Fig. 1*B*, *top left panel*). Conversely, monocytes derived from donors who did not express surface TLR1 exhibited no detectable signal above that observed with an isotype control Ab (Fig. 1*B*, *top right panel*). When cells were permeabilized before staining, intracellular TLR1 was detected in both groups in a diffuse pattern (Fig. 1*B*, *bottom panels*). However, monocytes that lacked cell surface TLR1 exhibited a greater intensity of intracellular staining that was distributed

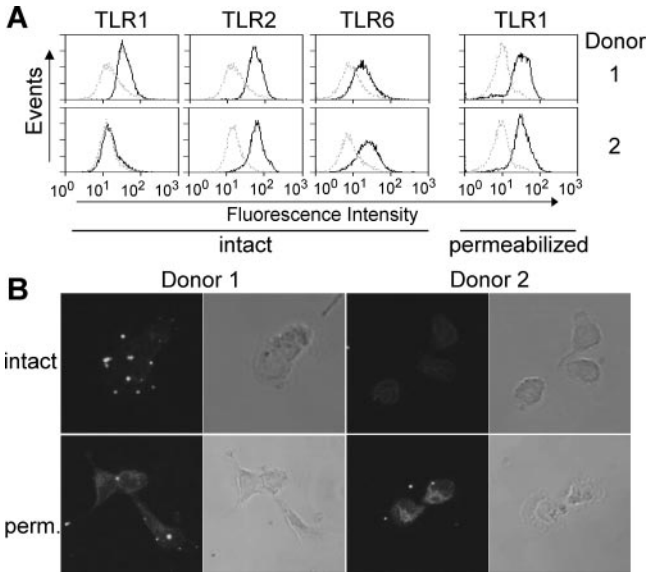

FIGURE 1. Surface, but not total, TLR1 expression levels vary among donors. *A*, Flow cytometry histograms showing expression of TLRs 1, 2, or 6 on PBMCs from a donor that expresses surface TLR1 (donor 1) and one that does not (donor 2). In the column on the *far right*, cells were fixed and permeabilized following isolation to allow for detection of intracellular TLR1. *B*, Confocal microscopy of monocytes from the same donors stained for TLR1. Cells were either untreated or fixed and permeabilized as indicated before Ab staining. A fluorescent field (*left*) with corresponding phase contrast field (*right*) is shown for each donor.

predominantly within the perinuclear region of the cell (Fig. 1*B*, *bottom right panel*). These results show that an inability to transport TLR1 to the cell surface underlies the deficiency in surface expression and suggests that instead the receptor accumulates within the cell, possibly within the endoplasmic reticulum or Golgi network.

Since no defect in surface expression of the phylogenetically related TLR6 molecule was observed in any donors, this suggested that a genetic polymorphism specific to the *TLR1* gene locus might underlie the lack of cell surface expression of TLR1. A search of the National Center for Biotechnology Information (NCBI) database revealed the existence of only two commonly occurring SNPs within *TLR1*, one of which, rs5743618, exchanges an isoleucine for a serine at amino acid position 602. This SNP was selected for further analysis as it is found at the junction of the transmembrane and intracellular domain of this receptor; a region that could affect intracellular trafficking. Additionally, amino acid sequence alignment of TLR1 from various vertebrate species revealed a highly conserved hydrophobic side chain (isoleucine, leucine, or valine) at this position that is also present in the closely related TLR6 and TLR10 receptors but is absent in TLR2 and all other TLR family members (data not shown).

We examined the cell surface expression of TLRs 1, 2, and 6 in peripheral blood monocytes obtained from 60 healthy volunteers and genotyped the blood donors with respect to the *TLR1 602* allele (Fig. 2*A*). One-way ANOVA revealed a highly significant difference in cell surface TLR1 expression among the genotypes ($p = 0.001$). Posthoc statistical tests demonstrated that significantly lower cell surface levels of TLR1 are expressed by monocytes derived from individuals who are homozygous for the *602S* allele in comparison to individuals who are either heterozygous ($p = 0.005$) or homozygous for the 602I allele ($p = 0.0005$). As expected, the levels of expression of either TLR2 or TLR6 were not found to be different between the three genotypes, demonstrating a lack of association between the *TLR1 602* allele and cell surface levels of these related receptors (Fig. 2*A*).

We next wished to determine whether the *602S* allele was associated with impaired inflammatory responses to *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)propyl]-(*R*)-cysteinyl-(lysyl)$_3$-lysine (Pam_3CSK_4), a synthetic triacylated lipopeptide agonist for the TLR1/2 heterodimer that mimics the stimulatory activity of natural bacterial lipoproteins (18, 19). In comparison to individuals who possess at least one *TLR1 602I* allele, monocytes obtained from individuals who are homozygous for *TLR1 602S* exhibited significantly lower levels of TNF-α release ($p < 0.05$) in response to Pam_3CSK_4. These results suggest that low cell surface levels of TLR1 impair cellular responses to this agonist (Fig. 2*B*). As expected, responses of monocytes to enteric bacterial LPS, an established agonist for TLR4, were not significantly different among the three genotypes.

Expression and function of TLR1 602S is impaired in heterologous systems

Since it remained possible that the *TLR1 602S* allele was not directly responsible for the TLR1 low phenotype but was simply in linkage disequilibrium with the true allele, we introduced this SNP into a TLR1 expression vector by site directed mutagenesis. While *TLR1 602I* was readily detected by flow cytometry on the surface of transfected cells, the *TLR1 602S* variant was not (Fig. 3*A*). However, total cellular levels of both variants of TLR1 were equally observed as revealed by flow cytometric analysis of permeabilized cells, as well as Western blot analysis of cell lysates (Fig. 3, *B* and *C*). To assess the functional

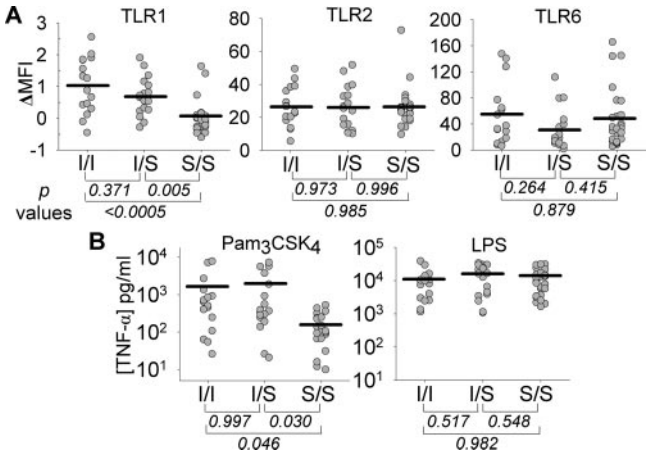

FIGURE 2. Individuals homozygous for the *602S allele* exhibit impaired surface expression and function of TLR1. *A*, Monocytes isolated from 60 blood donors were genotyped, and cell surface levels of TLRs 1, 2, and 6 were assessed by flow cytometry. Results are expressed as mean fluorescence intensity minus that of an isotype-matched Ab (ΔMFI). *B*, Monocytes from each blood donor were cultured for 16 h in the presence of a TLR2/1 agonist (10 ng/ml Pam_3CSK_4) or a TLR4 agonist (10 ng/ml LPS). TNF-α concentrations were then measured in the supernatants by ELISA. All scatter plots contain one data point for each donor, and the mean is indicated by the horizontal bar. Values of *p* were generated after one-way ANOVA using a Tukey posthoc test.

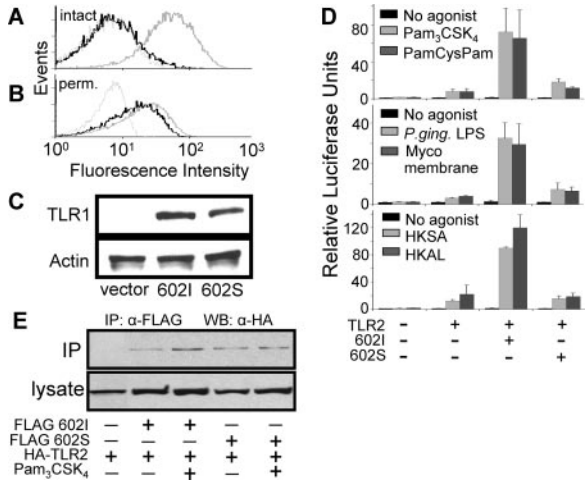

FIGURE 3. TLR1 602S, but not TLR1 602I, exhibits abrogated cell surface expression and responses to TLR2/1 agonists. HEK-293T cells were transfected with an expression plasmid containing either vector alone (dashed line), FLAG-TLR1 602I (solid gray line), or FLAG-TLR1 602S (solid black line). Cells were untreated (*A*) or permeabilized (*B*) before staining with labeled anti-FLAG Ab and analyzed by flow cytometry. Cells were also analyzed for FLAG-TLR1 expression by immunoblotting (*C*). *D*, SW620 cells were transfected as indicated along with an IL-8 promoter-driven luciferase gene. Forty-eight hours after transfection, cells were stimulated with the indicated TLR2/1 agonists, and luciferase activity was measured. The error bars represent the SD of three independent experimental values. *P. ging.* LPS, *Porphymonas gingivalis* LPS; Myco. Membrane, mycobacterial membrane fraction; HKSA, heat-killed *Staphylococcus aureus*; HKAL, heat-killed *Acholeplasma laidlawii*. *E*, 293T cells were co-transfected as indicated and stimulated for 5 min with 10 ng/ml Pam_3CSK_4 as indicated. Anti-FLAG M2 immunoprecipitates from cell lysates were separated and Western blotted using anti-HA Ab.

Table I. *The TLR1 602S allele is observed less frequently in Turkish leprosy patients than asymptomatic controls*

		Genotype *Hz (%)*					
	n	I/I	I/S	S/S	S Allele *Hz (%)*	Odds Ratio[a]	Confidence Interval (95%)[a]
Controls	90	37	41	22	43	0.48	0.29–0.80
Patients	57	56	35	9	26		
p Value [a]		0.02	0.59	0.02	0.004		

[a]Calculated by comparing patients and controls.

activity of the TLR1 602 variants, we used SW620 epithelial cells, a human colonic epithelial cell line that does not express either endogenous TLR1 or TLR2 and has been used to reconstitute the activity of this heterodimeric receptor pair (16). Following cotransfection with TLR2, the TLR1 602I variant mediated cellular responses to a variety of diverse agonists for this receptor pair, including a mycobacterial membrane preparation (Fig. 3*D*). In contrast, cells cotransfected with TLR2 along with the TLR1 602S variant did not mediate a response to any of these bacterial-derived components over that of TLR2 alone. Taken together with our previous findings, these results show that the *TLR1 602S* allele, and not a genetically linked allele, affects cell surface expression of this receptor as well as cellular responses to a variety of bacterial products. To our knowledge, this is the first demonstration of a TLR SNP that affects receptor trafficking.

TLRs 1 and 2 are believed to associate and form heterodimeric receptor complexes. Through coimmunoprecipitation experiments, we observed that both TLR1 602I and TLR1 602S variants associate with TLR2 in resting cells, presumably within an intracellular compartment such as Golgi apparatus (Fig. 3*E*). Following ligand stimulation, the active 602I variant coimmunoprecipitated additional TLR2, however, the deficient 602S variant did not (Fig. 3*E*). The signaling defect exhibited by TLR1 602S is entirely consistent with studies showing that TLRs 1 and 2 colocalize in the early phagosome during ingestion of yeast zymosan particles (20) and associate in lipid rafts within the plasma membrane following ligand stimulation (21, 22). Taken together, our results further support the idea that the defect in TLR1 602S signaling is due to aberrant trafficking to the plasma membrane where ligand induced interactions with TLR2 are required for signaling.

TLR1 602S is a common allele in Caucasians and protects against leprosy

Due to the established importance of TLR1 in mediating recognition of mycobacterial cell wall components, we assessed the frequency of the *I602S* allele in a cohort of 57 Turkish leprosy patients and 90 asymptomatic control patients recruited from centers in areas where this disease is endemic. Surprisingly, we found that the *602S* allele was significantly underrepresented in the leprosy patient population vs the control population with a statistically significant odds ratio of 0.48 (Table I). When we examined our data in the context of genotype frequencies, an association between the homozygous *602I* genotype ($p = 0.018$) and leprosy was observed while a decreased incidence of leprosy was associated with the homozygous 602S genotype ($p = 0.017$) (Table I). Our results strongly suggest that diminished TLR1 expression and function is protective for the development of clinical leprosy, suggesting that *M. leprae* subverts the TLR system to evade the host immune system. In this regard, mycobacterial-induced TLR2 activation has been shown to render macrophages unresponsive to IFN-γ, a cytokine that would otherwise induce phagolysosomal killing, Ag presentation, and T cell recruitment responses (23, 24). We are currently investigating whether the *TLR1 602S* allele protects against mycobacteria by enabling macrophages to maintain their responsiveness to IFN-γ during infection.

Table II. *The frequency of the TLR1 602 allele varies considerably among different races*[a]

Race	*n*	I/I	I/S	S/S
White	66	7.5	35	58
African descent	27	56	37	7
East Asian descent	21	100	0	0

[a]Each value represents the percentage of individuals of a given race with the indicated *TLR1 602* genotype. Frequencies were derived from the NCBI SNP database, as well as the results of this study.

As the TLR1/2 heterodimer mediates cellular responses to numerous components of bacterial, fungal, and leptospiral origin, the *I602S* SNP has implications for a number of infectious diseases. For example, the SNP *P753Q* within the TLR2 signaling domain has been associated with impaired responsiveness to extracts of *Borrelia burgdorferi*, yet homozygosity for this allele is observed at a significantly lower frequency in patients with Lyme disease compared with healthy controls (25). In conjunction with our findings, we propose that, while impaired TLR function is detrimental for the host in the context of acute infection, it may be beneficial in the context of chronic conditions such as Lyme disease or leprosy.

A phylogenetic analysis of TLRs identified in all vertebrate species to date shows that all family members, including the subfamily comprising TLRs 1, 6, and 10, have evolved under strong purifying selection (4, 5). Genotyping reveals that *602S* is the most prevalent allele found in white individuals (75% frequency), with a decreased allele frequency observed in individuals of Turkish (43%) and African descent (26%) (Table II). Overall, the data indicate that more than half of white persons are homozygous for the *602S* allele. Surprisingly, all 21 donors of East Asian ancestry genotyped in our study were homozygous for the *602I* allele, and the *602S* allele was never observed in this group. These results suggest that, if a strong purifying selection for a particular *602* allele took place, it was restricted by either additional genetic or environmental factors, for example, specific pathogens or past epidemics that were constrained geographically. In the context of mycobacterial infection, a study of 25,000 nursing home residents revealed that African Americans were twice as likely to develop tuberculosis as Caucasians and that no identifiable social or environmental factor could account for this difference (26). This distribution is consistent with the idea that the *602I* allele is a predisposing genetic factor

for this disease. *TLR1 I602S* represents the most common SNP affecting TLR function identified in any population to date. Additional studies will be required to shed light on the biological role of this polymorphism and its evolutionary selection in the context of infectious disease and chronic inflammation.

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Disclosures

The authors have no financial conflict of interest.

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