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CUTTING EDGE

Cutting Edge: A Toll-Like Receptor 2 Polymorphism That Is Associated with Lepromatous Leprosy Is Unable to Mediate Mycobacterial Signaling[1]

Pierre-Yves Bochud, Thomas R. Hawn,*† and Alan Aderem[2]*†*

***Toll-like receptors (TLRs) are key mediators of the innate immune response to microbial pathogens. We investigated the role of TLRs in the recognition of* Mycobacterium leprae *and the significance of TLR2Arg^{677}Trp, a recently discovered human polymorphism that is associated with lepromatous leprosy. In mice, TNF-α production in response to* M. leprae *was essentially absent in TLR2-deficient macrophages. Similarly, human TLR2 mediated* M. leprae*-dependent activation of NF-κB in transfected Chinese hamster ovary and human embryonic kidney 293 cells, with enhancement of this signaling in the presence of CD14. In contrast, activation of NF-κB by human TLR2Arg^{677}Trp was abolished in response to* M. leprae *and* Mycobacterium tuberculosis*. The impaired function of this TLR2 variant provides a molecular mechanism for the poor cellular immune response associated with lepromatous leprosy and may have important implications for understanding the pathogenesis of other mycobacterial infections. The Journal of Immunology,* 2003, 170: 3451–3454.**

Toll-like-receptors (TLRs)[3] mediate the innate immune recognition of microbial pathogens and shape development of the adaptive immune response (1–4). Human (h)TLRs are type I transmembrane proteins with an extracellular leucine-rich repeat domain and an intracellular domain homologous to the IL-1R (Toll-IL receptor (TIR) domain) (5). Upon stimulation, the TIR domain binds to an adapter protein, MyD88, which in turn, through a series of intermediate molecules, causes nuclear translocation of NF-κB and activation of transcription of proinflammatory cytokines (6). These cytokines play an essential role in the host innate immune response and determine the activation of adaptive immune mechanisms.

TLRs confer specificity to the innate immune response through their extracellular domain. TLR4 is responsible for the recognition of LPS (7), and TLR5, for bacterial flagellin (8). TLR2 has been shown to mediate the innate immune response to ligands derived from a variety of pathogens, including Gram-positive bacteria, *Mycoplasma*, yeasts, and parasites (3, 6). In addition, both *Mycobacterium tuberculosis* and *Mycobacterium avium* stimulate cells through TLR2 (9–11). The importance of TLRs in human diseases has recently been shown in studies of polymorphisms in TLR4. Two missense mutations (Asp^{299}Gly and Thr^{399}Ile) affecting the extracellular domain of hTLR4 are associated with hyporesponsiveness to LPS (12) and an increased incidence of Gram-negative septic shock (13). A polymorphism situated in the TIR domain of hTLR2 (Arg^{753}Gln) has been detected, although its clinical significance has not yet been elucidated (14).

Leprosy, or Hansen's disease, is a chronic and debilitating disease that annually affects >700,000 new individuals as reported by the World Health Organization (15). Leprosy is characterized by a large spectrum of clinical manifestations that depend on the host cell-mediated immune response against the pathogen (16). At one pole of the disease, tuberculoid leprosy patients manifest a strong cellular immune response, resulting in few, localized, often self-healing paucibacillary lesions. At the opposite pole, lepromatous leprosy patients have a limited cellular immune response, leading to a disseminated disease, involving extended multibacillary lesions of the skin and nerves. The factors that influence which type of leprosy develops are not well understood. Yet, epidemiologic studies clearly indicate that susceptibility to leprosy has a significant genetic component (17). A recent study showed that a mutation in the intracellular domain of hTLR2 (Arg^{677}Trp) is associated with lepromatous leprosy in a Korean population. The Arg^{677}Trp hTLR2 mutation was found in 10 of 45 lepromatous leprosy patients (22%), whereas it was not observed in 41 tuberculoid leprosy patients nor in 45 healthy controls (18). Of note, the Arg residue at position 677, which is conserved among human and mouse TLRs (TLRs 1–9), is situated in close proximity to

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; TIR, Toll-IL receptor; h, human; SNP, single nucleotide polymorphism; HEK, human embryonic kidney; CHO, Chinese hamster ovary; MLcw, *Mycobacterium leprae* cell wall fraction components; MTBcw, *Mycobacterium tuberculosis* cell wall fraction components; Pam_3CSK_4, synthetic tripalmitoylated lipopeptide; PGL-1, phenolic glycolipid-1; WT, wild type; ELAM-1, endothelial leukocyte molecule-1; RLU, relative luciferase unit.

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the locus corresponding to the dominant-negative mutations of the mouse *TLR4* and *TLR2* gene (Pro^{681}His) (7, 19).

In this study, we investigated whether *Mycobacterium leprae* activates the innate immune system through TLR2 and whether the Arg^{677}Trp single nucleotide polymorphism (SNP) affects signaling. We demonstrate that TLR2 is necessary to mediate responsiveness to *M. leprae*. Furthermore, we show that the Arg^{677}Trp mutation abrogates the ability of TLR2 to mediate a response to *M. leprae* as well as *M. tuberculosis* stimulation.

Materials and Methods

Cells and reagents

Human embryonic kidney (HEK)293 (ATCC no. CRL-1573; American Type Culture Collection, Manassas, VA) and Chinese Hamster Ovary (CHO) K1 (ATCC no. CCL-61; American Type Culture Collection) cells were maintained at 37°C in a 5% CO_2 incubator in DMEM (HEK) or Ham's F12 medium (BioWhittaker, Walkersville, MD) (CHO), both containing 10% heat-inactivated FCS, 1% L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Polymixin B was from Sigma-Aldrich (St. Louis, MO).

Irradiated *M. leprae*, phenolic glycolipid-1 (PGL-1), and *M. leprae* SDS-soluble cell wall fraction components (MLcw) were provided by P. Brennan (Colorado State University, Fort Collins, CO) through the Leprosy Research Support contract (NO1-AI-55262; National Institutes of Health, National Institute of Allergy and Infectious Diseases). *M. tuberculosis* cell wall fraction components (MTBcw) were obtained from J. Belisle (Colorado State University) through the TB Research Materials and Vaccine Testing contract (NO1-AI-75320; National Institutes of Health, National Institute of Allergy and Infectious Diseases). LPS from *Salmonella Minnessota* R595 Re was obtained from List Biological Laboratories (Campbell, CA) and *Saccharomyces cerevisiae* particle zymosan from Molecular Probes (Eugene, OR). Bacterial flagellin was extracted from *Salmonella typhimurium* TH4778 (*fliB*$^-$, *fliC*$^+$) as previously described (8). Synthetic tripalmitoylated lipopeptide (Pam_3CSK_4) was from EMC Microcollections (Tuebingen, Germany) and peptidoglycan (*Staphylococcus aureus*) from Fluka (Buchs, Switzerland). Levels of contaminating LPS as determined by the chromogenic *Limulus* amebocyte lysate test (BioWhittaker) were 0.02, 0.007, 0.0005, and 0.0001 endotoxin U/ml, in wells incubated with zymosan, irradiated *M. leprae*, MTBcw, and MLcw, respectively. These values correspond to ~2, 0.7, 0.05, and 0.01 pg/ml endotoxin, respectively. PGL-1 had no detectable endotoxin.

Bone marrow macrophages

The mutant mouse strains deficient in TLR2 and TLR4 have been described previously (20, 21). The TLR2-deficient mice were backcrossed two times with C57BL/6. The TLR4-deficient mice have been backcrossed six times with C57BL/6 mice and wild-type (WT) littermates were used as controls. Bone marrow-derived macrophages were obtained from femoral bone marrow cells that were cultured for 6–9 days in complete DMEM with 20% L929 fibroblast (ATCC no. CCL-1; American Type Culture Collection) conditioned medium. Subsequently, cells were plated in a 96-well dish at 2×10^4/well and stimulated for 24 h. The concentration of TNF-α in supernatant was determined by ELISA (R&D Systems, Minneapolis, MN). The University of Washington's Institutional Animal Care and Use approved all animal protocols.

DNA expression vectors

hTLR2 was amplified from THP1 cDNA (ATCC no. TIB-202; American Type Culture Collection) by using primers 5′-ATGCCACATACTTTGTGG-3′ and 5′-GGACTTTATCGCAGCTCTCAG-3′ and cloned into pEF6/V5-His-TOPO (Invitrogen, San Diego, CA). The TLR2 mutation described in Korean patients (Arg^{677}Trp) (18) and the dominant-negative mutant (Pro^{681}His) (19) were elaborated using Quick Change site-directed mutagenesis (Stratagene, La Jolla, CA). The *hTLR2* gene cloned into pEF6/V5-His-TOPO (WT) was amplified by PCR using primers 5′-AAGTTGTGTCTGCACAAGTGGGACTTCATTCC-3′ and 5′-GGAAGTTCAACACAGACGTGTTCACCCTGAAGTAA-3′ to generate the Arg^{677}Trp mutant and primers 5′-GACTTCATCCATGGCAAGTGG-3′ and 5′-CGCCCTGAAGTAGGTACCGTTCACCTAG-3′ to generate the Pro^{681}His mutant. *Dpn*I digestion was used to remove methylated DNA before transformation into competent *Escherichia coli*. The template and both mutants were verified by full-gene sequencing. The CD14 and hTLR5 expression constructs have been described previously (8).

Luciferase assay

The day before transfection, CHO and HEK293 cells were plated in a 96-well dish at 2×10^4 or 6×10^4 per well, respectively. Transient transfection was performed using the Polyfect transfection reagent (Qiagen, Chatsworth, CA). Cells were transfected with 0.25–0.5 μg of endothelial leukocyte molecule-1 (ELAM-1) firefly luciferase construct, 0.025–0.05 μg of the thymidine kinase *Renilla* luciferase (pRL-TK) vector (Promega, Madison, WI), and 0.25–0.5 μg of the expression vector(s) per well. CHO cells were washed in PBS 2–3 h after transfection. Twenty hours after transfection, cells were stimulated for 4 h. The cells were lysed in passive lysis buffer and the luciferase activity was determined using the dual-luciferase reporter assay system (Promega).

Results

TLR2 mediates innate immune response to M. leprae

We examined the role of TLR2, TLR4, and TLR5 in the inflammatory response to *M. leprae*. First, we analyzed the production of TNF-α in bone marrow macrophages from WT and TLR2- and TLR4-deficient mice upon *M. leprae* stimulation. Bone-marrow macrophages were cultured with irradiated *M. leprae*, MLcw, or LPS for 24 h, and the concentration of TNF-α was measured in the supernatant by ELISA. WT and TLR4-deficient macrophages produced similar amounts of TNF-α in response to MLcw and MTBcw. In TLR2-deficient macrophages, the level of TNF-α produced upon mycobacterial stimulation was dramatically reduced (*M. leprae*) or not detectable (MTBcw) (Fig. 1*A*). WT and TLR2-deficient macrophages produced similar amounts of TNF-α in response to LPS, whereas TLR4-deficient macrophages produced no detectable TNF-α (20). As a control, assays were also performed with polymixin B (at 10 μg/ml) to inhibit any possible effect of LPS contamination. However, no differences were observed when polymixin B was present.

The role of TLR5 in *M. leprae*-induced signaling was examined in transiently transfected CHO cells, a cell line that does not express functional endogenous TLR2 (22). CHO cells were transfected with a construct expressing hTLR5 together with an

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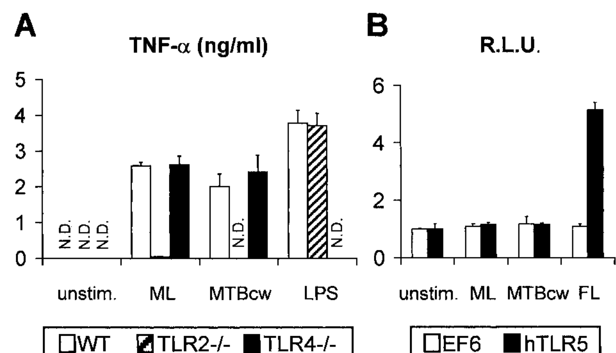

FIGURE 1. Innate immune recognition of dead *M. leprae* cells is mediated by TLR2, but not TLR4 or TLR5. *A*, Bone marrow macrophages from WT and TLR2- and TLR4-deficient mice were cultured in the presence of irradiated *M. leprae* (ML; 80 μg/ml), MTBcw (1 μg/ml), and LPS (10 ng/ml) for 24 h. TNF-α production was measured by ELISA. The results are mean ± SD of triplicate wells and are representative of three different experiments. ND, Not detected. *B*, CHO cells were transiently transfected with a vector expressing hTLR5 or an empty vector (EF6), together with NF-κB reporter (ELAM-1 firefly luciferase) and transfection control (thymidine kinase *Renilla* luciferase). Cells were stimulated with irradiated *M. leprae* (80 μg/ml), MTBcw (1 μg/ml), and bacterial flagellin (FL; 5 ng/ml), and luciferase activity was measured with a luminometer. Firefly luciferase units were divided by *Renilla* units to obtain relative luciferase units (RLU). The results are mean ± SD of triplicate wells and are representative of four separate experiments.

NF-κB reporter (ELAM-1), and a transfection control (pRL-TK). The cells were stimulated with irradiated *M. leprae* cells, MTBcw, and bacterial flagellin as a positive control (8). Expression of hTLR5 did not confer *M. leprae* nor MTBcw sensitivity (Fig. 1*A*). As a control, flagellin responsiveness was detected in CHO cells transfected with hTLR5.

These data suggest that the innate immune recognition of irradiated *M. leprae* cells is mediated by TLR2, but not TLR4 or TLR5. To confirm the role for hTLR2 in *M. leprae*-induced signaling, we transiently transfected CHO cells with a construct expressing hTLR2, together with a luciferase reporter as described previously. The transfected cells were then stimulated with irradiated *M. leprae* cells, MLcw, or PGL-1, a specific *M. leprae* Ag that was recently reported to be directly involved in the neuropathogenesis of leprosy (23, 24). Expression of hTLR2 was sufficient to confer responsiveness to *M. leprae* and MLcw, but not to PGL-1 (Fig. 2*A*). Because CD14 is required for the recognition of certain TLR2 ligands (25–27), we transfected HEK293 cells with hTLR2 together with a construct expressing CD14 as shown in Fig. 2*B*. HEK293 cells were stimulated with MLcw, PGL-1, and yeast particles (zymosan). Expression of hTLR2 was sufficient to confer responsiveness to MLcw in HEK293 cells, and coexpression of CD14 together with hTLR2 facilitated this response. As a control, coexpression of CD14 was required to activate NF-κB production upon zymosan stimulation. Expression of hTLR2 with or without CD14 did not confer PGL-1 responsiveness.

Polymorphism in hTLR2 affects the innate immune response to mycobacteria

To evaluate the relevance of the hTLR2 SNP described in Korean patients with lepromatous leprosy ($Arg^{677}Trp$), we transfected HEK293 cells with constructs expressing WT and mutant ($Arg^{677}Trp$) hTLR2. As a control, we used a construct expressing another hTLR2 mutant ($Pro^{681}His$) analogous to a mouse TLR2 dominant-negative mutant (19). Transiently transfected HEK293 cells were stimulated with escalating doses of MTBcw (Fig. 3*A*) and MLcw (Fig. 3*B*). HEK293 cells transfected with WT hTLR2 induced NF-κB activation in a dose-dependent fashion in response to MLcw and MTBcw. In contrast, NF-κB activation was abolished in HEK293 cells transfected with the Korean ($Arg^{677}Trp$) and dominant-negative ($Pro^{681}His$) hTLR2 mutants. All the transfectants expressed equivalent amounts of V5 epitope-tagged hTLR2 as revealed on Western blot (Fig. 3*C*). Transfection of CD14 together with hTLR2 $Arg^{677}Trp$ did not confer responsiveness to MLcw (Fig. 3*D*). Of note, $Arg^{677}Trp$ and $Pro^{681}His$ hTLR2 mutants also failed to activate NF-κB in response to Pam_3CSK_4, peptidoglycan, and zymosan.

Discussion

In this paper, we show that TLR2 plays an essential role in the innate immune response to *M. leprae* and that an hTLR2 polymorphism ($Arg^{677}Trp$) that was associated with lepromatous leprosy in Korea is unable to respond to *M. leprae* stimulation.

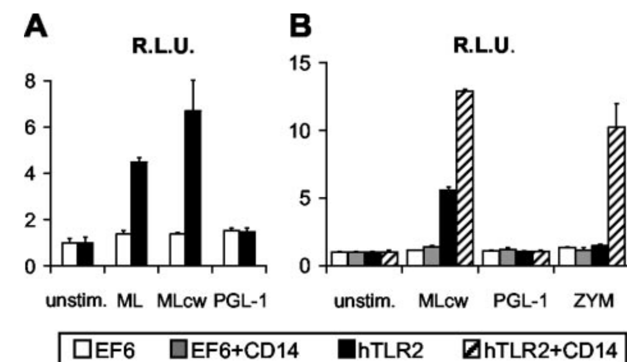

FIGURE 2. Expression of hTLR2 is sufficient to mediate *M. leprae* and MLcw-induced NF-κB activation. *A*, CHO cells were transiently transfected with a vector expressing hTLR2 or an empty vector (EF6) as described in Fig. 1*B*. Cells were stimulated with *M. leprae* (80 μg/ml), MLcw (1 μg/ml), and PGL-1 (1 μg/ml). The results expressed in RLU are mean ± SD of triplicate wells and are representative of four different experiments. *B*, HEK293 cells were transiently transfected as described in Fig. 2. Expression of hTLR2 together with CD14 was compared with expression of hTLR2 alone. The total amount of DNA per well was normalized by addition of EF6. Cells were stimulated with MLcw (1 μg/ml), PGL-1 (1 μg/ml), and the yeast particle zymosan (ZYM, 400 ng/ml). The results are expressed in RLU and represent mean ± SD of triplicate wells. Similar results were obtained in three different experiments.

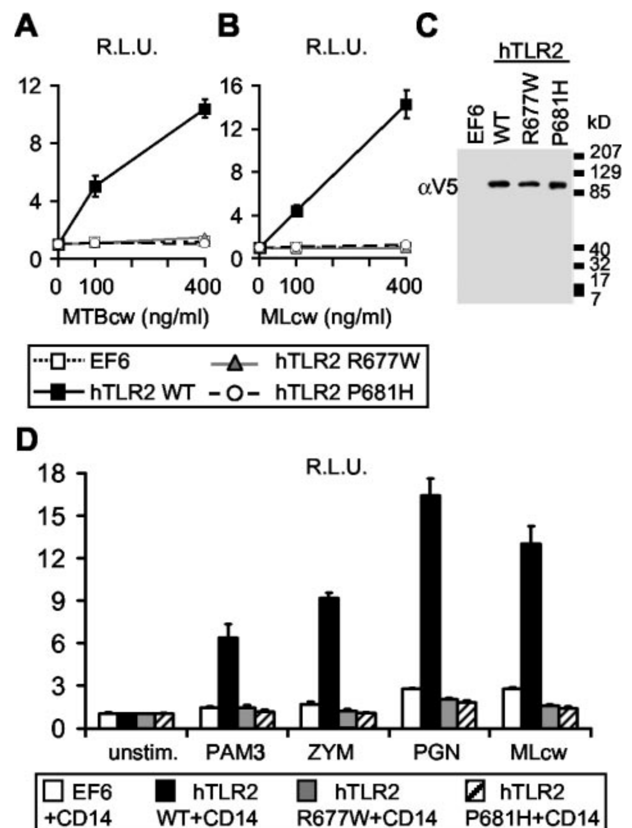

FIGURE 3. $Arg^{677}Trp$ polymorphism in hTLR2 affects NF-κB activation in response to *M. leprae* and *M. tuberculosis*. HEK293 cells were transiently transfected with constructs expressing WT, mutant ($Arg^{677}Trp$; $Pro^{681}His$) hTLR2 (0.5 μg each), or an empty vector (EF6) together with an NF-κB reporter as described in Fig. 1*B*. Cells were stimulated with escalating doses of MTBcw (*A*) and MLcw (*B*), as indicated. Results are expressed in RLU and represent mean ± SD of triplicate wells. Similar results have been observed in two separate experiments. *C*, Anti-V5 Western blot of the lysates shown in *A* and *B*. Equal volumes of lysate were loaded on a gel and blotted with anti-V5 Abs. *D*, HEK293 cells were transiently transfected as described in *A* and *B*. In addition, CD14 was added in each well. Cells were stimulated with Pam_3CSK_4 (PAM3; 400 ng/ml), zymosan (ZYM; 400 ng/ml), peptidoglycan (PGN; 50 μg/ml), and MLcw (1 μg/ml). Results, expressed in RLU, are mean ± SD of triplicate wells and are representative of three separate experiments.

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The fact that TLR2 plays a central role of in the innate immune recognition of *M. leprae* and MLcw is not unexpected. TLR2 recognizes ligands derived from various infectious agents, including Gram-positive bacteria, yeasts, and parasites (reviewed in Refs. 3 and 6). Different mycobacterial products are recognized by TLR2, including the soluble tuberculosis factor (a short-term culture filtrate of *M. tuberculosis* containing phosphatidylinositolmannan) (28, 29), the 19-kDa protein (a secreted Ag of *M. tuberculosis*) (30, 31), and lipoarabinomannan purified from rapidly growing avirulent mycobacteria (9). Both live *M. avium* and live *M. tuberculosis* are known to activate innate immune cells through TLR2 (9, 10). Our data show that the cell surface molecule CD14 enhances but is not required to mediate *M. leprae*-induced NF-κB activation through TLR2. Similarly, CD14 has been shown to increase TLR2-mediated response to *M. tuberculosis* (19) and the 19-kDa protein by ~2-fold (30).

We found that TLR4 is not necessary for the innate immune recognition of irradiated *M. leprae*. In contrast, live *M. tuberculosis*, but not live *M. avium*, uses both TLR4 and TLR2 (9). Recently, a study in C3HeJ mice suggested a protective role of TLR4 against chronic infection with *M. tuberculosis* in vivo (32). A role for TLR4 in the recognition of live *M. leprae* in vivo cannot be excluded by the current studies. Our findings show that TLR5 does not mediate *M. leprae* signaling. The only TLR5 ligand that has been identified so far is flagellin, the major component of bacterial flagella (8), a protein that is not known to be present in mycobacteria.

We found that the expression of the $Arg^{677}Trp$ variant of hTLR2, which was identified in Korean patients with lepromatous leprosy, was not able to mediate NF-κB activation in response to MLcw and MTBcw. This finding supports the epidemiologic data observed in Korea. Although the results from our reconstitution system show clear signaling differences between WT and mutant TLR2, it will be important to confirm these results in primary cells from individuals that carry the $Arg^{677}Trp$ polymorphism. $Arg^{677}Trp$ is the first TLR2 SNP that has been associated with a clinical disease that is unable to mediate signaling. This nonfunctional SNP variant highlights a possible role for the innate immune response in the pathogenesis of leprosy and other mycobacterial infections. Because the $Arg^{677}Trp$ mutation is situated in the intracellular signaling domain of hTLR2, it is not surprising that it also impairs the response to other TLR2 ligands, like Pam_3CSK_4, peptidoglycan, and zymosan. Thus, $Arg^{677}Trp$ mutation in hTLR2 may influence the innate immune response to other pathogens that are recognized by TLR2, and may have important implications for understanding the pathogenesis of a wide range of infections.

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