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

Pierre-Yves Bochud,* Thomas R. Hawn,*† and Alan Aderem2*†

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 TLR2Arg677Trp, a recently discovered 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 TLR2Arg677Trp 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) 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-negative 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 (Asp299Gly and Thr399Ile) 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 (Arg75Gln) 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 (Arg677Trp) is associated with lepromatous leprosy in a Korean population. The Arg677Trp 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...
the locus corresponding to the dominant-negative mutations of the mouse TLR4 and TLR2 gene (Pro6His) (7, 19).

In this study, we investigated whether Mycobacterium leprae activates the innate immune system through TLR2 and whether the Arg677Trp single nucleotide polymorphism (SNP) affects signaling. We demonstrate that TLR2 is necessary to mediate responsiveness to M. leprae. Furthermore, we show that the Arg677Trp 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% CO2 incubator in DMEM (HEK) or Ham’s F12 medium (BioWhittaker, Walkerville, MD) (CHO), both containing 10% heat-inactivated FCS, 1% l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. PolyB (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) 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). 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). 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). 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). 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). 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). 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).

Bacterial flagellin was expressed and purified as described (8). Synthetic tripalmitoylated lipopeptide (Pam3CSK4) was from Molecular Probes (Eugene, OR). Bacterial flagellin was expressed and purified as described (8). Synthetic tripalmitoylated lipopeptide (Pam3CSK4) was from Molecular Probes (Eugene, OR). Limulus amebocyte lysate test (BioWhittaker) was used to detect endotoxin.

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 (Fig. 1A). 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 empty vector (EF6).

Luciferase assay

The day before transfection, CHO and HEK293 cells were plated in a 96-well dish at 2 × 104 or 6 × 104 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 endotoxin 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).

DNA expression vectors

hTLR2 was amplified from THP1 cDNA (ATCC no. TIB-202; American Type Culture Collection) by using primers 5’-ATGCCAATATTT GTGCC-3’ and 5’-GGACTTTTATGCAGCTCAG-3’ and cloned into pEF6/VS-His-TOPO (Invitrogen, San Diego, CA). The TLR2 mutation described in Korean patients (Arg677Trp) (18) and the dominant-negative mutant (Pro6His) (19) were elaborated using Quick Change site-directed mutagenesis (Stratagene, La Jolla, CA). The hTLR2 gene cloned into pEF6/VS-His-TOPO (WT) was amplified by PCR using primers 5’-AAGTTTGGTCTG CAAAAGTGTTGCAACTTCC-3’ and 5’-GGAGGTGTCCACAGACG TGTTTCACCGAAGAAT-3’ to generate the Arg677Trp mutant and primers 5’-GACTTCTCATCGGCGAATTG-3’ and 5’-GGCCCTGGAGT ACCAGGTTCTCCAG-3’ to generate the Pro6His mutant. 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).

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. The concentration of TNF-α in supernatant was determined 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. 1A). 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 (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 empty vector (EF6). Transient transfection was performed using the Polyfect transfection reagent (Qiagen, Chatsworth, CA). Cells were transfected with 0.25–0.5 μg of endotoxin 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).
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. 1A). 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 the neuropathogenesis of leprosy (23, 24). Expression of hTLR2 was sufficient to confer responsiveness to MLcw, PGL-1, and yeast particles (zymosan). Expression of hTLR2 was sufficient to confer responsiveness to MLcw (Fig. 2A). HEK293 cells were transfected with MLcw, PGL-1, and yeast particles (zymosan). Expression of hTLR2 was sufficient to confer responsiveness to MLcw together with hTLR2 facilitated this response. As a control, coexpression of CD14 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 (Arg677Trp), we transfected HEK293 cells with constructs expressing WT and mutant (Arg677Trp) hTLR2. As a control, we used a construct expressing another hTLR2 mutant (Pro681His) analogous to a mouse TLR2 dominant-negative mutant (19). transiently transfected HEK293 cells were stimulated with escalating doses of MTBcw (Fig. 3A) and MLcw (Fig. 3B). 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 (Arg677Trp) and dominant-negative (Pro681His) hTLR2 mutants. All the transfectants expressed equivalent amounts of V5 epitope-tagged hTLR2 as revealed on Western blot (Fig. 3C). Transfection of CD14 together with hTLR2 Arg677Trp did not confer responsiveness to MLcw (Fig. 3D). Of note, Arg677Trp and Pro681His hTLR2 mutants also failed to activate NF-κB in response to Pam3CSK4, 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 (Arg677Trp) 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. 1B. 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. 2A. 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.** Arg677Trp 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 (Arg677Trp; Pro681His) hTLR2 (0.5 μg each), or an empty vector (EF6) together with an NF-κB reporter as described in Fig. 1B. 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 Pam3CSK4 (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.
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 tuberculous 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 findings 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<sup>677</sup>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 reconstruction 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<sup>677</sup>Trp polymorphism. Arg<sup>677</sup>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<sup>677</sup>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 Pam3CSK₄, peptidoglycan, and zymosan. Thus, Arg<sup>677</sup>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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References

A note from the author: The reference list contains important works that contribute to our understanding of the innate immune system and its role in mycobacterial infections. These references are cited to provide a comprehensive view of the research on TLR2 and its role in the immune response to mycobacterial infections. The references include works from various authors and institutions, highlighting the collaborative nature of scientific research in the field. The references also provide a basis for further exploration and investigation into the role of TLR2 in the immune response to mycobacteria, which is crucial for understanding the pathogenesis of mycobacterial infections and the development of effective therapeutic strategies.