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Evolution of an intronic microsatellite polymorphism in Toll-like receptor 2 among primates

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Abstract Nonhuman primates express varying responses to *Mycobacterium tuberculosis*: New World monkeys appear to be resistant to tuberculosis (TB) while Old World monkeys seem to be particularly susceptible. The aim of this study was to elucidate the presence of the regulatory guanine–thymine (GT) repeat polymorphisms in intron 2 of Toll-like receptor 2 (*TLR2*) associated with the development of TB in humans and to determine any variations in these microsatellite polymorphisms in primates. We sequenced the region encompassing the regulatory GT repeat microsatellites in intron 2 of *TLR2* in 12 different nonhuman primates using polymerase chain reaction amplification, TA cloning, and automatic sequencing. The nonhuman primates included for this study were as follows: chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), Celebes ape (*Macaca nigra*), rhesus monkey (*Macaca mulatta*), pigtail macaque (*Macaca nemestrina*), patas monkey (*Erythrocebus patas*), spider monkey (*Ateles geoffroyi*), Woolly monkey (*Lagothrix lagotricha*), tamarin (*Saguinus labiatus*), and ring-tailed lemur (*Lemur catta*). Nucleotide sequences encompassing the regulatory GT repeat region are similar across species and are completely conserved in great apes. However, Old World monkeys lack GT repeats altogether, while New World monkeys and ring-tailed lemurs have much more complex structures around the position of the repeats. In conclusion, the genetic structures encompassing the regulatory GT repeats in intron 2 of human *TLR2* are similar among nonhuman primates. The sequence is most conserved in New World monkeys and less in Old World monkeys.

Keywords Toll-like receptor 2 · Microsatellite polymorphism · Evolution · Primates

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Introduction

The human Toll-like receptors (TLRs) are pattern recognition molecules, which play important roles in early innate immune recognition and inflammatory responses (Hoffmann et al. 1999; Kopp and Medzhitov 1999; Medzhitov and Janeway 1997a,b, 2000). In addition to their critical roles in modulating innate immunity, TLRs are essential in the orientation of the adaptive immune response through the induction of the Th-1 immune response (Schnare et al. 2001). Among the ten human TLRs, *TLR2* plays a key role in the immune responsiveness to peptidoglycans (Takeuchi et al. 1999; Yoshimura et al. 1999), lipoteichoic acid of Gram-positive bacteria (Schwandner et al. 1999), enterotoxin from *Escherichia coli* (Hajishengallis et al. 2005), mycobacterial lipoarabi-

nomannan (Underhill et al. 1999; Wieland et al. 2004), phosphatidylinositol (Jones et al. 2001), and leptospiral lipopolysaccharide (Werts et al. 2001). This crucial role of *TLR2* could explain the evolutionary conservation of the sequence of the *TLR2* gene in chicken (*Gallus gallus*), mouse (*Mus musculus*), Japanese flounder (*Paralichthys olivaceus*), zebra fish (*Danio rerio*), and human (*Homo sapiens*) (Boyd et al. 2001; Chaudhary et al. 1998; Hirono et al. 2004; Jault et al. 2004; Rock et al. 1998).

High susceptibility to *Mycobacterium tuberculosis* infection of *TLR2*-deficient mice (Drennan et al. 2004; Reiling et al. 2002) suggests that mutations affecting *TLR2* expression may impair host response to this pathogen. Recently, we reported the presence of guanine–thymine (GT) repeat polymorphisms in intron 2 of human *TLR2* (NM_ 003264, located at 4q32) and the association of the number of GT repeats with the development of tuberculosis (TB) in Koreans (Yim et al. 2004, 2006).

Nonhuman primates express varying responses to *M. tuberculosis*. New World monkeys appear to be more resistant to *M. tuberculosis* because they show proliferating lesions with giant cells with less extensive caseation. In contrast, Old World monkeys seem to be particularly susceptible to *M. tuberculosis*, showing a severe and fatal form of TB when exposed (Scott 1992). In addition, the genus *Macaca* appears to be especially susceptible and exhibits the most human-like response (Good 1968; West et al. 1981; Ribi et al. 1971). The natural variations in TB infection rates implying variations in susceptibility among nonhuman primates were 0.4% out of 20,580 imported cynomolgus monkeys (*Macaca fascicularis*), 0.5% out of 1,621 imported rhesus monkeys (*Macaca mulatta*), but none out of 712 imported African green monkeys (*Cercopithecus aethiops) (Centers for Disease Control and Prevention 1993).*

Because the coding sequence of *TLR2* is well conserved through the species (Boyd et al. 2001; Hirono et al. 2004; Jault et al. 2004), we aimed to elucidate the presence of the regulatory GT repeats polymorphism in intron 2 of *TLR2* and to document any variations in this microsatellite polymorphism in primates, which might correlate with *TLR2* expression and susceptibility to mycobacterial infection.

Materials and methods

Primates

We obtained 19 different nonhuman primate DNA samples from the Coriell Institute for Medical Research (Camden, NJ, USA). These samples consisted of: one chimpanzee (*Pan troglodytes*), one bonobo (*Pan paniscus*), one gorilla (*Gorilla gorilla*), one orangutan (*Pongo pygmaeus*), two Celebes ape (*Macaca nigra*), three rhesus monkey (*M. mulatta*), three pigtail macaque (*Macaca nemestrina*), two patas monkey (*Erythrocebus patas*), one spider monkey (*Ateles geoffroyi*), one woolly monkey (*Lagothrix lagotricha*), one tamarin (*Saguinus labiatus*), and two ring-tailed lemur (*Lemur catta*).

Amplification of sequences including GT repeats

We amplified a region of about 200 bp encompassing the GT repeat microsatellite with primers designed for human *TLR2*: 5′-gcattgctgaatgtatcaggga-3′ and 5′-caccatccacaaag tatgtggc-3′. Polymerase chain reaction (PCR) amplifications were carried out using a thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA, USA) in PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton® X-100), 100 μM of each dNTP, 25 μM primers, and 1 U of *Taq* polymerase (Promega, Madison, WI, USA) in a final volume of 50 μl. After an initial denaturation step at 95°C for 5 min, samples were subjected to 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. This temperature regimen was followed by a final elongation step at 72°C for 10 min. The reaction products of the PCR amplifications and enzyme restrictions were analyzed by electrophoresis on 3% agarose gels.

TA cloning and sequencing

After cutting the gel containing the DNA fragments from each species, we extracted DNA fragment with QIAquick gel extraction kit using a microcentrifuge (QIAGEN, Valencia, CA, USA). Using these purified DNA samples, we performed TA cloning and transformed the plasmid into *E. coli* using TOPO TA Cloning and One Shot Chemical Transformation using Chemically Competent *E. coli* and following the manufacture's protocols (Invitrogen, Carlsbad, CA, USA). After amplification of the plasmid, we extracted the DNA fragments using Wizard Plus SV Minipreps (Promega) following a standard centrifugation protocol. PCR products were sequenced using an ABI 377 sequencer (Applied Biosystems) according to the manufacturer's protocols.

Phylogenetic analysis

To perform an alignment of about 200-bp-long sequences encompassing GT repeats from each species and a subsequent phylogenetic analysis, we used a multiple alignment algorithm provided by ClustalW software (Thompson et al. 1994). The parameters for the present analysis were as follows: gap open penalty, 15.00; gap

extension penalty, 6.66; delay divergent sequences, 30%; DNA transitions weight, 0.50; protein weight matrix, Gonnet series; DNA weight matrix, IUB; use negative matrix, OFF; toggle residue-specific penalties, ON; toggle hydrophilic penalties, ON; hydrophilic residues, GPSNDQEKR; gap separation distance, 4; toggle end gap separation, OFF.

Results

Sequences encompassing the repeat regions in individual primate species

PCR with primers designed based on the human sequence for *TLR2* was successful in all primates even though only the chimpanzee (*P. troglodytes*) had a published sequence (XM_526707, chromosome 4) showing similarity to that of the human *TLR2* gene. In humans, the number of GT repeats is known to vary between 12 and 28, as determined by the studies derived from people with diverse ancestral origins (Yim et al. 2004). The nucleotide sequences encompassing the repeat region were similar across species (Fig. 1). All great apes analyzed in our study (chimpanzee, bonobo, gorilla, and orangutan) have essentially the same sequence around GT repeats with that of human. Four Old World monkeys (Celebes ape, rhesus monkey, pigtail macaque, and patas monkey) have almost the same sequence but lack GT repeats and the first seven nucleotides following the repeats. Prosimians (ring-tailed lemur) and New World monkeys (spider monkey, woolly monkey, and tamarin) showed distinct variations from those of humans suggesting a long evolutionary divergence.

Variations in GT repeats between individual primate species

The GT repeats are completely conserved in great apes, although the range of such repeat numbers in each species should be clarified by analyzing more individuals. Old World monkeys lack any GT repeats, and two New World monkeys, the spider monkey and the Tamarin, have much more complex structures at these positions (Fig. 1). The ring-tailed lemur has a sequence closer to that of humans than Old World monkeys despite being more phylogenetically distant.

Phylogenic tree from sequences around GT repeats

Figure 2a shows the phylogenic tree generated from primates based on DNA evidence complemented by fossil evidence (Goodman et al. 1998). In contrast, Fig. 2b was generated based on the about 200-bp-long sequences encompassing the GT repeats in intron 2 of *TLR2* using the ClustalW program (see "Materials and methods"). The polymorphic nature of even the very short sequences around the GT repeats may well reflect the overall evolutionary distances among primates suggested by the fossil evidence.

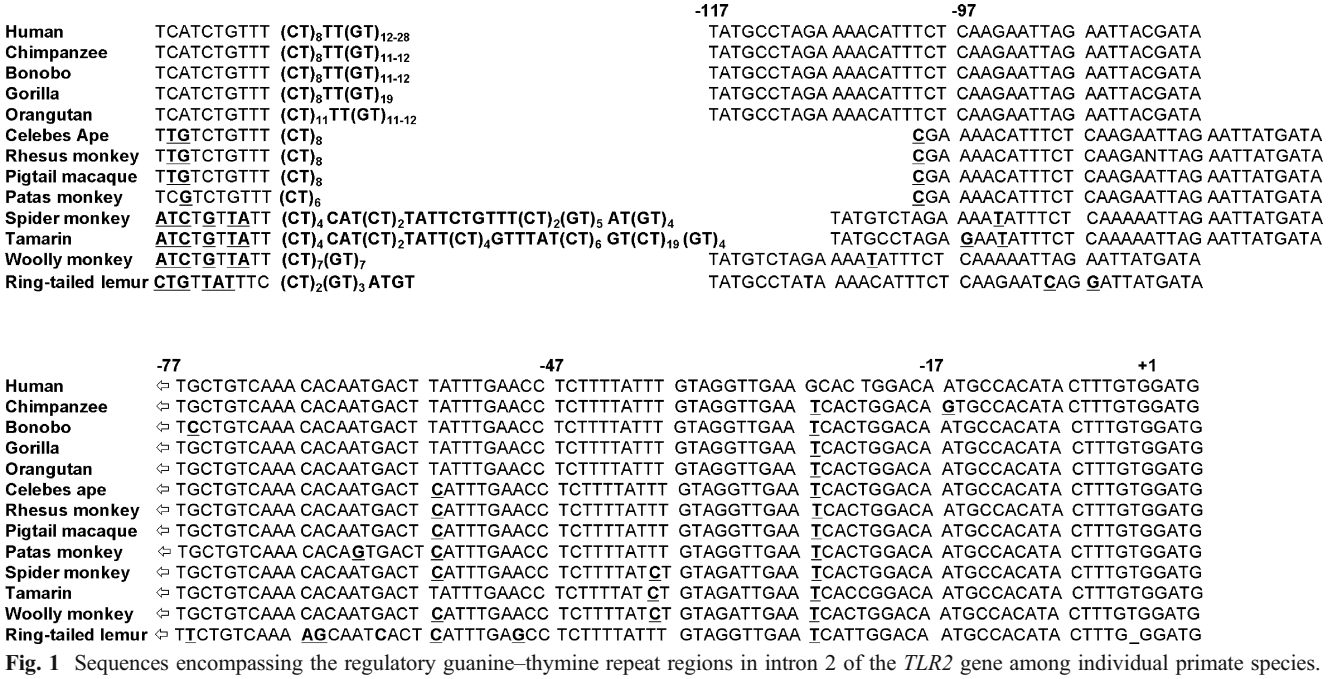

Fig. 1 Sequences encompassing the regulatory guanine–thymine repeat regions in intron 2 of the *TLR2* gene among individual primate species. '+*1*' represents the translation initiation site

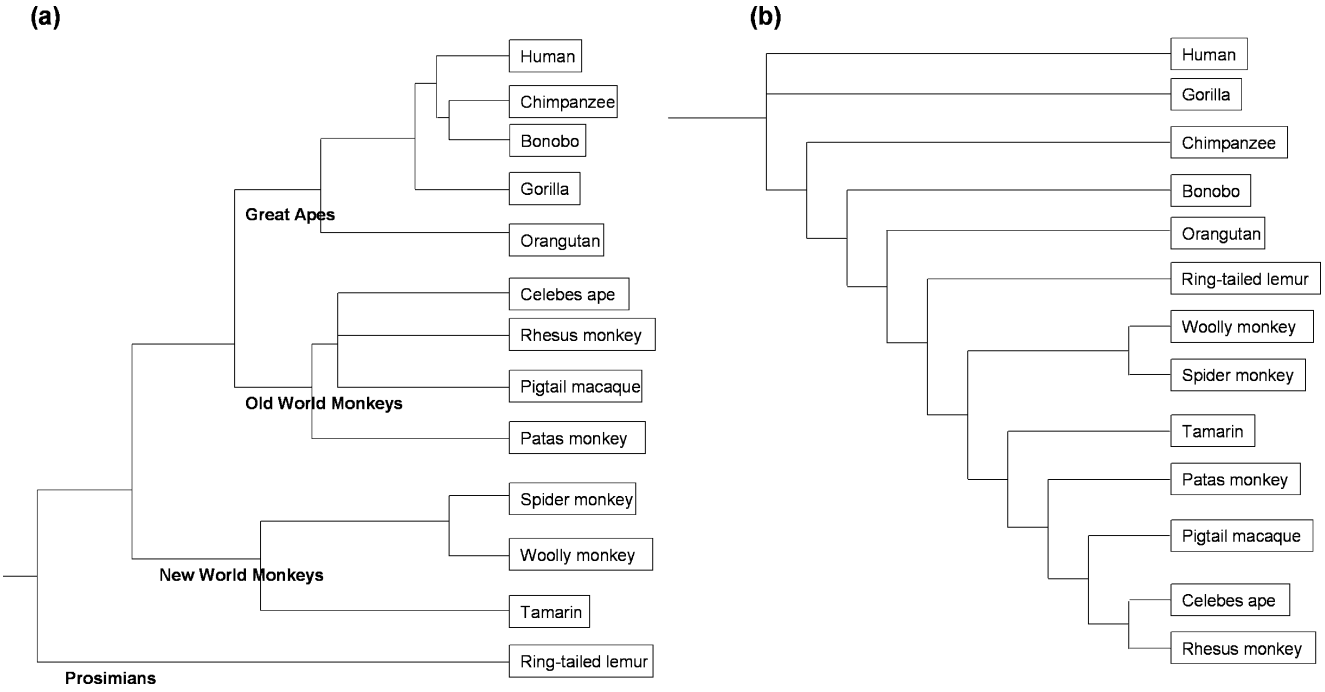

Fig. 2 Phylogenetic trees generated from primate data based on **a** DNA evidence complemented by fossil evidence; and **b** DNA evidence based on the 185 bases including the regulatory guanine–thymine repeats in intron 2 of *TLR2*. Line length does not denote phylogenic distance

Discussions

Microsatellites are short tandem repeats of two to five nucleotide repeat pairs that are generally fewer than 30 repeats (Tautz and Schlotterer 1994). They are highly variable regions of DNA with heterozygosity levels often exceeding 70% (Webster et al. 2002). They are often found in noncoding regions, making them of interest to those studying evolution (Zhu et al. 2000). Several theories have been advanced for the cause of these microsatellites, including replication slippage which causes the addition or subtraction of one or only a few repeats (Levinson and Gutman 1987). However, recent work suggests that the process may be more complex (Ellegren 2000).

We recently reported microsatellite polymorphisms consisting of variable numbers of GT repeats in intron 2 of human *TLR2* (Yim et al. 2004), a receptor that plays an important role in host immunity against *M. tuberculosis* infection (Drennan et al. 2004; Reiling et al. 2002). Through molecular and genetic studies involving patients with TB, we determined that shorter GT repeats were associated with weaker promoter activity, lower expression of the *TLR2* on the surface of human monocytes, and development of clinical TB (Yim et al. 2006). Therefore, at both in vitro and in vivo levels, these polymorphisms exert a regulatory effect. Although the numbers of these repeats were variable, *TLR2* intron 2 GT repeats were never absent from hundreds of individual human samples from different races (Yim et al. 2004, 2006).

Based on the long-standing observation that nonhuman primates have widely variable immune responses to *M. tuberculosis* (Good 1968; Scott 1992), we hypothesized that the regulatory GT intronic repeats in the *TLR2* gene might be informative and different among nonhuman primates. We found that the sequences around the GT repeats were relatively conserved among these species. As expected, the greater the phylogenic distance of primates from humans, the greater the variations observed in the GT repeats, except for the ring-tailed lemur. It is interesting to note that repeats were not observed among those Old World monkeys particularly susceptible to *M. tuberculosis* (Scott 1992), although these variations could not be directly connected to increased susceptibility among these monkeys. However, the functional implications of these variations can now be elucidated by inserting these sequences into the recombinant promoter intron 2 of *TLR2*/reporter construct previously described (Yim et al. 2004).

Given the high level of variation in the numbers of these regulatory GT intronic repeats in *TLR2* in humans and their functional importance, species level variations of the sequences encompassing the repeats among other primates could be important. Although deeper understanding of these findings will require both more individuals from each species, as well as careful expression studies within and between members and species, the fact that these sequences show variation in overall structure between humans and other primates is noteworthy. Given that this region of *TLR2* has a regulatory role in humans, it is likely that it

functions in the same way in other primates. That suggests that the evolutionary forces impacting on *TLR2* and its functions in humans and other primates have been quite active. Since nontuberculous mycobacteria are ubiquitous and have existed for millenia, *Mycobacterium bovis* has been recovered from New World bison from about 16,000 years ago (Rothschild et al. 2001), and tuberculosis has been an endemic infection in humans for several thousand years; it is likely that mycobacteria have been interacting with the mammalian genome over a long period. The fact that there is such broad variation in such a small region in one of the critical immune response genes for tuberculosis and other mycobacteria suggests that this is a region that is under intense pressure. Because the variation we identified was in a regulatory region and not in the coding sequence, it suggests that fine tuning of host response to external stimuli, such as bacterial products, has been and remains important. The fact that among humans we retain high- and low-responding alleles suggests that these alterations may carry benefits and detriments, depending on the stimulus.

The genetic structures encompassing the regulatory GT repeats in intron 2 of the *TLR2* gene are well conserved among nonhuman primates. These variations may have bearing on susceptibility to *M. tuberculosis* in primates as they do in humans.

References

Boyd Y, Goodchild M, Morroll S, Bumstead N (2001) Mapping of the chicken and mouse genes for toll-like receptor 2 (TLR2) to an evolutionarily conserved chromosomal segment. Immunogenetics 52:294–298

Centers for Disease Control and Prevention (1993) Tuberculosis in imported nonhuman primates-United States, June 1990-May 1993. MMWR Morb Mortal Wkly Rep 42:572–576

Chaudhary PM, Ferguson C, Nguyen V, Nguyen O, Massa HF, Eby M, Jasmin A, Trask BJ, Hood L, Nelson PS (1998) Cloning and characterization of two Toll/Interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans. Blood 91:4020–4027

Drennan MB, Nicolle D, Quesniaux VJ, Jacobs M, Allie N, Mpagi J, Fremond C, Wagner H, Kirschning C, Ryffel B (2004) Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. Am J Pathol 164:49–57

Ellegren H (2000) Heterogeneous mutation processes in human microsatellite DNA sequences. Nat Genet 24:400–402

Good RC (1968) Biology of the mycobacterioses. Simian tuberculosis: immunologic aspects. Ann N Y Acad Sci 154:200–213

Goodman M, Porter CA, Czelusniak J, Page SL, Schneider H, Shoshani J, Gunnell G, Groves CP (1998) Toward a phylogenetic classification of primates based on DNA evidence complemented by fossil evidence. Mol Phylogenet Evol 9:585–598

Hajishengallis G, Tapping RI, Martin MH, Nawar H, Lyle EA, Russell MW, Connell TD (2005) Toll-like receptor 2 mediates cellular activation by the B subunits of type II heat-labile enterotoxins. Infect Immun 73:1343–1349

Hirono I, Takami M, Miyata M, Miyazaki T, Han HJ, Takano T, Endo M, Aoki T (2004) Characterization of gene structure and expression of two toll-like receptors from Japanese flounder, Paralichthys olivaceus. Immunogenetics 56:38–46

Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA (1999) Phylogenetic perspectives in innate immunity. Science 284:1313–1318

Jault C, Pichon L, Chluba J (2004) Toll-like receptor gene family and TIR-domain adapters in Danio rerio. Mol Immunol 40:759–771

Jones BW, Means TK, Heldwein KA, Keen MA, Hill PJ, Belisle JT, Fenton MJ (2001) Different Toll-like receptor agonists induce distinct macrophage responses. J Leukoc Biol 69:1036–1044

Kopp EB, Medzhitov R (1999) The Toll-receptor family and control of innate immunity. Curr Opin Immunol 11:13–18

Levinson G, Gutman GA (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol Biol Evol 4:203–221

Medzhitov R, Janeway CA Jr (1997a) Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 9:4–9

Medzhitov R, Janeway CA Jr (1997b) Innate immunity: the virtues of a nonclonal system of recognition. Cell 91:295–298

Medzhitov R, Janeway C Jr (2000) Innate immune recognition: mechanisms and pathways. Immunol Rev 173:89–97

Reiling N, Holscher C, Fehrenbach A, Kroger S, Kirschning CJ, Goyert S, Ehlers S (2002) Cutting edge: Toll-like receptor (TLR) 2- and TLR4-mediated pathogen recognition in resistance to airborne infection with Mycobacterium tuberculosis. J Immunol 169:3480–3484

Ribi E, Anacker RL, Barclay WR, Brehmer W, Harris SC, Leif WR, Simmons J (1971) Efficacy of mycobacterial cell walls as a vaccine against airborne tuberculosis in the Rheusus monkey. J Infect Dis 123:527–538

Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF (1998) A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA 95:588–593

Rothschild BM, Martin LD, Lev G, Bercovier H, Bar-Gal GK, Greenblatt C, Donoghue H, Spigelman M, Brittain D (2001) Mycobacterium tuberculosis complex DNA from an extinct bison dated 17,000 years before the present. Clin Infect Dis 33:305–311

Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R (2001) Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2:947–950

Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ (1999) Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem 274:17406–17409

Scott GBD (1992) Comparative primate pathology. Oxford University Press, Oxford

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 11:443–451

Tautz D, Schlotterer C (1994) Simple sequences. Curr Opin Genet Dev 4:832–837

Thompson J, Higgins D, Gibson T (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Underhill DM, Ozinsky A, Smith KD, Aderem A (1999) Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc Natl Acad Sci USA 96:14459–14463

Webster MT, Smith NG, Ellegren H (2002) Microsatellite evolution inferred from human-chimpanzee genomic sequence alignments. Proc Natl Acad Sci USA 99:8748–8753

Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A,

Tobias PS, Ulevitch RJ (2001) Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol 2:346–352

West CS, Vainisi SJ, Vygantas CM, Beluhan FZ (1981) Intraocular granulomas associated with tuberculosis in primates. J Am Vet Med Assoc 179:1240–1244

Wieland CW, Knapp S, Florquin S, de Vos AF, Takeda K, Akira S, Golenbock DT, Verbon A, van der Poll T (2004) Non-mannose-capped lipoarabinomannan induces lung inflammation via toll-like receptor 2. Am J Respir Crit Care Med 170:1367–1374

Yim JJ, Ding L, Schaffer AA, Park GY, Shim YS, Holland SM (2004) A microsatellite polymorphism in intron 2 of human Toll-like receptor 2 gene: functional implications and racial differences. FEMS Immunol Med Microbiol 40:163–169

Yim JJ, Lee HW, Lee HS, Kim YW, Han SK, Shim YS, Holland SM (2006) The association between microsatellite polymorphisms in intron II of the human Toll-like receptor 2 gene and tuberculosis among Koreans. Genes Immun 7:150–155

Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D (1999) Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol 163:1–5

Zhu Y, Queller DC, Strassmann JE (2000) A phylogenetic perspective on sequence evolution in microsatellite loci. J Mol Evol 50:324–338