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Transcriptional Regulation of the Human Toll-Like Receptor 2 Gene in Monocytes and Macrophages

Viola Haehnel,* Lucia Schwarzfischer,* Matthew J. Fenton,† and Michael Rehli2*

This report investigates the molecular basis for tissue-restricted and regulated expression of the pattern recognition receptor Toll-like receptor (TLR)2 in human monocytes and macrophages. To define the proximal promoter, the full 5'-sequence and transcriptional start sites of TLR2 mRNA were determined. The human TLR2 gene was found to consist of two 5' noncoding exons followed by a third coding exon. Alternative splicing of exon II was detected primarily in human blood monocytes. The proximal promoter, exon I, and part of intron I were found to be located in a CpG island. Although CpG methylation of the proximal human TLR2 promoter in cell lines correlated with TLR2 repression, the promoter was unmethylated in primary cells, indicating that CpG methylation does not contribute to the cell-type specific expression of human TLR2 in normal tissues. The promoter sequence contains putative binding sites for several transcription factors, including Sp1 and Ets family members. Reporter gene analysis revealed a minimal promoter of 220 bp that was found to be regulated by Sp1, Sp3, and possibly PU.1. Interestingly, no sequence homology was detected between human and murine TLR2 promoter regions. In contrast to murine TLR2, expression of human TLR2 in monocytes/macrophages is not induced by the proinflammatory stimuli LPS or macrophage-activating lipopeptide-2, and reporter activity of the promoter was not enhanced by stimuli-induced NF-κB activation in THP-1 or MonoMac-6 cells. Our findings provide an initial definition of the human TLR2 promoter and reveal profound differences in the regulation of an important pattern recognition molecule in humans and mice. The Journal of Immunology, 2002, 168: 5629–5637.

In vertebrate organisms the innate branch of the immune system greatly relies on the recognition of conserved microbial structures (pathogen-associated molecular patterns (PAMPs)). Recent studies have demonstrated an import role of members of the Toll-like receptor (TLR) family in the initiation of intracellular signaling pathways through the recognition of PAMPs (1–3). Only five years ago, the prototypic Toll protein of Drosophila—a transmembrane receptor which was initially shown to control dorsal-ventral patterning in the embryo—was also found to be required for antifungal responses in adult flies (4). Since then, 10 mammalian Toll homologs have been identified that contain a leucine-rich ectodomain and a conserved cytoplasmic domain shared with both chains of the IL-1R, the IL-18R, SIGRR, and MyD88, the so-called Toll/IL-1R homologous region. Both genetic and biochemical data support a common signaling pathway that finally leads to the activation of NF-κB (1–3).

Several studies using either TLR knockout mice or TLR mutant mouse strains have shown that mammalian TLR proteins are able to recognize specific microbial structures. Signaling cascades activated by LPS, the abundant glycolipid of the outer membrane of Gram-negative bacteria, are initiated through TLR4 (5–7). Another PAMP, bacterially derived CpG DNA, is recognized through a TLR9-dependent mechanism (8). TLR5 recognizes bacterial flagellin from both Gram-positive and Gram-negative organisms (9). The protein product of the TLR2 gene has been implicated in signal transduction events induced by several microbial products (e.g., peptidoglycans, lipopolypeptides, and lipoxarinomannans) (10–17). In contrast to other TLR proteins, TLR2 seems to recognize microbial patterns as a heterodimer, e.g., with TLR6 or maybe TLR1 (18). In mice, destructive mutations of TLR2 impede a normal response to lipopolypeptides and cause a high susceptibility to Gram-positive infection. Murine TLR2 macrophages show normal responses to LPS stimulation (14). Although the genetic evidence described above strongly supports the contention that TLR4 is the predominant, if not exclusive, receptor for LPS, an additional role of TLR2 as LPS receptor is still debated. TLR2 does appear to mediate cellular activation by purified LPS from Leptospira interrogans and Porphyromonas gingivalis, which are structurally different from enteric LPS (19, 20).

Expression of TLR2 in humans is restricted to a small number of cell types, including predominantly myelomonocytic cells (monocytes, macrophages, dendritic cells, and granulocytes) (21, 22). Both the basal level of TLR2 expression and its inducible regulation may influence responses to microbial infection. Accordingly, we have sought to characterize the human TLR2 promoter and to analyze those factors that govern TLR2 gene expression in human monocytes/macrophages. Interestingly, the expression of human and murine TLR2 genes is controlled by distinct, nonconserved regulatory elements. We have found that promoter sequences of both species show no significant homology and, in contrast to its murine counterpart, the human TLR2 promoter does not respond to microbial pattern-activated NF-κB in monocytes and macrophages.

Materials and Methods

Chemicals

All chemical reagents were purchased from Sigma-Aldrich (Berlin, Germany) unless otherwise noted. Protease inhibitors are from Roche Biochemicals (Basel, Switzerland). Oligonucleotides were synthesized by TIB
Molbiol (Berlin, Germany). Antiseras for supershift analyses were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells
PBMCs were separated by leukapheresis of healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque (American Red Cross, Philadelphia, PA). Monocytes were isolated from PBMC by countercurrent centrifugal elutriation in a J6-M Elutri (Beckman, München, Germany) as previously described. Monocytes were >90% pure as determined by morphology and expression of CD14 Ag. Isolated monocytes were cultured in low-endotoxin RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with vitamins, antibiotics, pyruvate, and nonessential amino acids, plus 10% FCS (Life Technologies). The human monocytic cell line MonoMac-6 was grown in MEM supplemented with vitamins, antibiotics, pyruvate, and nonessential amino acids, plus 10% FCS (Life Technologies). The human monocytic cell line MonoMac-6 was grown in complete RPMI medium as above, with the addition of 1% OPI media supplement. The human cervical carcinoma cell line HeLa was maintained in DMEM plus 10% FCS. Where indicated, cells were treated with 100 ng/ml purified LPS from Salmonella abortus equi (a gift from C. Galanos, Max-Planck-Institut, Freiburg, Germany) or with 400 U/ml of the myco-plasmaindole macrophage-activating lipopeptide (MALP)-2 (a gift from P. Mühlethaler, Gesellschaft für Biotechnologische Forschung, Braun-sbach, Germany). The latter was kept as a stock solution of 1 mg/ml (4 × 107 M) in water-2-propanol (1:1, v/v) at 4°C and was diluted as described. Drosophila S2 Schneider cells (a gift from Dr. W. Falk, Internal Medicine I, University Hospital, Regensburg, Germany) were cultivated in Schneider’s Drosophila medium (Invitrogen, Karlsruhe, Germany).

RNA preparation, RT-PCR, and Northern analysis
Total RNA was isolated from different cell types by the guanidine thio-yanate/acid phenol method (23). Two micrograms of total RNA from either cell type was reverse transcribed using oligo(dT) primer and Superscript II (Life Technologies). Primer positions and sizes for the amplified fragments of TLR2 and β-actin are indicated (see Fig. 2). PCR conditions were optimized to assure that the amplification was still exponential at the indicated cycle numbers. The amplified products were sequenced to confirm their identity. For Northern analysis, total RNA (10 μg/lane) was separated by electrophoresis on 1% agarose/formaldehyde gels and transferred to nitrocellulose filters. The membranes were hybridized with a [32P]-labeled cDNA probe of a 1500-bp restriction fragment of human TLR2 (random primed labeling by Hart-and Analytics, Braunschweig, Germany). Autoradiography was performed at −70°C and bands were scanned with a personal densitometer (Molecular Dynamics, Sunnyvale, CA).

DNA sequence analysis
The cDNA sequencing was done by Dye Deoxy Terminator Cycle Sequencing (PE Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions and sequences were analyzed on the PE Applied Biosystems DNA Sequence System (model 373A).

Transient DNA transfections
MonoMac-6 cells were transfected using DEAE-dextran. A total of 5 × 105 MonoMac-6 cells per milliliter were seeded into tissue culture flasks 24 h before transfection. On the next day, 6 ml cell suspension was washed twice with suspension TBS (STBS) solution (25 mM TrisCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na2HPO4, 0.7 mM CaCl2, and 0.5 mM MgCl2) and pelleted. One microgram of reporter plasmid and 62.5 ng renilla control vector were mixed with DEAE-dextran (400 μg/ml) in 130 μl STBS buffer and immediately added to the pelleted MonoMac-6 cells. The cells were incubated at 37°C for 20 min, washed twice with STBS, resuspended, and cultured in complete RPMI 1640 medium. Where indicated, LPS (100 ng/ml) or MALP-2 (400 μg/ml) was added for the cultures h 4 before harvesting. The cell lines THP-1 and HeLa were transfected as previously described (24). The transfected cell lines were cultured for 48 h and harvested, and cell lysates were assayed for firefly and renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity of individual transfections was normalized against renilla luciferase activity. Drosophila S2 Schneider cells were transfected using Effectene reagent (Qiagen) according to the manufacturer’s instructions. Briefly, 4 × 105 Schneider cells were cells were cotransfected using 10 μl Effectene reagent and 1.5 μg total DNA (1 μg of reporter plasmid, 0.25 μg of individual expression plasmids). Duplicate transfections were harvested after 48 h and cell lysates were assayed for firefly luciferase activity using the Luciferase Reporter Assay System (Promega). Firefly luciferase activity of individual transfections was normalized against protein concentration measured using a BCA assay (Sigma-Aldrich).

Nuclear extracts and EMSA
Nuclear extracts were prepared with a variation of the method of Osborn et al. (17). All buffers used contained 1 mM Na3VO4 and a mixture of protease inhibitors (2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml chymostatin, and 5 μg/ml leupeptin; Sigma). Twenty micrograms of nuclear protein were fractionated on 7% polyacrylamide gels. Double-stranded oligonucleotides were labeled with [α-32P]deoxycty- tide 5′-triphosphate using Klenow DNA polymerase. A double-stranded oligonucleotide containing the proximal Sp1 motif from the human TLR2 promoter (Thr2-Sp) as well as a consensus Sp1 motif (5′-CGATCCGCCCGGCAG-3′) were used as cold competitor. The binding reaction contained 2.5 μg of nuclear extract protein, 0.5 μg of poly(dI/dC) (Pharmacia, 300 μg/ml) as the carrier, 100 μM each of dATP, dGTP, dCTP, and dTTP, 10 μM dithiothreitol, 50 mM KCl, 1 mM DTT, 50 mM Hepes (pH 7.9), 5% glyceral, and 20 nmol of probe DNA in a final volume of 10 μl. Antiseras used in supershift analyses were added after 15 min and samples were loaded onto polyacrylamide gels after standing at room temperature for a total of 30 min. Buffers and running conditions used have been described. Gels were fixed in 5% acetic acid, dried, and autoradiographed.

DNA preparation and bisulfite sequencing
Genomic DNA from various cell types was prepared using the Blood and Cell Culture DNA Midi kit from Qiagen. Modification of DNA with sodium bisulfite (25) was performed as follows. A total of 5 μg genomic DNA in 50 μl 10 mM TrisCl, pH 8.0, 1 mM EDTA were denatured with 5.5 μl NaOH (3 M) at 37°C for 15 min. After the addition of 540 μl sodium bisulfite (3.8 M) and 15 μl hydrochioride (0.4 M), samples were mixed, divided into six aliquots, and covered with mineral oil. Incubation was continued for 3 h in a PCR cycler at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min (25). Samples were recombined after treatment and DNA was recovered using Wizard (Promega) in 100 μl H2O and desulfonated by the addition of 11 μl NaOH (3 M) and subsequent incubation at 37°C for 15 min. The
DNA was then precipitated using ammonium acetate and ethanol and resuspended in 50 μl 10 mM Tris-Cl, pH 8.0, 1 mM EDTA. A total of 5 μl of DNA was amplified in individual nested PCR for both strands using the following primers: sense strand (first round), Ss1 (5′-GGTTTAAAGAAAAATTTGGTTGGG-3′) and Sas1 (5′-CAAAACTAAAACCCTAACA-3′); sense strand (second round), Ss2 (5′-TTTGTGAATTAGTTTTTTTGGTGG-3′) and Sas2 (5′-AAAAACTCRAACAAATCACC-3′); antisense strand (first round), As1 (5′-CTTCTCATAATTACCAAGCTCC-3′) and Asas1 (5′-TAGGAAGGGGTAGAGAGATT-3′); antisense strand (second round), As2, (5′-CAACCCCAACTCTTCTTCTCC-3′) and Asas2 (5′-AGAATTYGTAGTTATTTATGAGAGA-3′). Products from the second round of nested PCR were precipitated and sequenced. Unmethylated cytosine residues are converted into thymidine residues during the bisulfite treatment and subsequent PCR amplification, whereas methylated cytosine residues are protected.

**Results**

**Isolation of full-length TLR2 transcripts and structure of the human TLR2 gene**

As an initial step to characterize regulatory regions of the human TLR2 gene, we determined the transcriptional start sites and the full-length sequence of TLR2 transcripts in human monocytes, which express relatively high levels of TLR2 mRNA. PCR fragments of full-length sequence of TLR2 transcripts in human monocytes, TLR2 (5′-CTGTTTAAGAAAAATTGGTTGGG-3′) and Sas1 (5′-CAAAACTAAAACCCTAACA-3′); sense strand (first round), Ss2 (5′-TTTGTAAAGAAAAATTTGGTG-3′) and Sas2 (5′-AAAAACTCRAACAAATCACC-3′); antisense strand (first round), As1 (5′-CTTCTCATAATTACCAAGCCTCC-3′) and Asas1 (5′-TAGGAAGGGGTAGAGAGATT-3′); antisense strand (second round), As2 (5′-CAACCCCAACTCTTCTTCTCC-3′) and Asas2 (5′-AGAATTYGTAGTTATTTATGAGAGA-3′). Products from the second round of nested PCR were precipitated and sequenced. Unmethylated cytosine residues are converted into thymidine residues during the bisulfite treatment and subsequent PCR amplification, whereas methylated cytosine residues are protected.

**Alternative splicing of the human TLR2**

The majority of 5′ fragments amplified from monocytes by RLM-RACE-PCR was found to lack the sequence of exon II, indicating the presence of alternatively spliced forms of TLR2. To analyze the presence of splicing forms in different TLR2-expressing cell types, RT-PCR with specific primers flanking the region between exons I and III of the human TLR2 gene was performed. As shown in Fig. 2A, the obtained band pattern varied among cell types and individuals. With the exception of monocytes, all cell types primarily expressed the longest transcript containing exon I, II, and III. In monocytes three distinct products were detectable; however, the relative distribution of transcripts was dependent on the individual donor. Sequencing of cloned fragments from monocytes revealed five different splicing variants and the usage of two alternative splice acceptor sites and three splice donor sites in exon II (see Fig. 1B). All splice junctions contained the expected GT splice donor and AG splice acceptor. The shortest fragment completely lacked exon two, and the three intermediate fragments were using acceptor site I/donor site I, acceptor site II/donor site II, or acceptor site III/donor site II (see Fig. 1B). All splicing variants contained the complete sequence of exon III and are predicted to code for one TLR2 protein. Interestingly, splicing of TLR2 mRNAs rapidly changed upon adherence of freshly isolated monocytes. As shown for two individual donors in Fig. 2B, the ratio of short and long splice variants is altered during monocyte differentiation in vitro, with the long splice variant being predominant after adherence of monocytes for only 3 h.

**Activity of the proximal human TLR2 promoter in myeloid THP-1 and nonmyeloid HeLa cells**

Published Northern blot analyses suggest that human TLR2 is predominantly expressed in spleen, lung, and peripheral blood leukocytes (26, 27). Monocytes, monocyte-derived dendritic cells, and granulocytes were identified as the major TLR2-expressing cells in human blood (21, 22).

To further analyze mechanisms regulating myeloid expression of TLR2, we cloned fragments of the 5′ proximal promoter region of the human TLR2 gene, ranging from 2.7 kb to 100 bp upstream of the ATG-start codon, into a luciferase reporter plasmid (Fig. 3). Transient transfection analysis was performed in the monocytic cell line THP-1 and nonmyeloid cell line HeLa (cervical carcinoma). Luciferase activities were normalized for transfection efficiency by cotransfection with a renilla construct, and results for individual cell lines were compared relative to the activity of a CMV promoter-driven construct that was used as a positive control. As shown in Fig. 3, deletion analysis localizes a region directing maximal reporter gene expression in THP-1 cells to ~220 bp proximal to the major transcriptional start site. Significant reporter activity was also measured in HeLa cells, which do not express TLR2 (data not shown), indicating that additional regulatory elements are involved in cell type-specific expression of the human TLR2 gene.

**The proximal promoter region of human TLR2 is distinct from murine TLR2**

The 5′ proximal region of human TLR2 lacks TATA boxes or consensus initiator sequences. Instead it contains several GC-rich regions that are often found in housekeeping genes and may determine transcriptional initiation (Fig. 1B). Within the ~220 bp region, putative binding sites for Sp1 family transcription factors and an E-box element were identified using computational analysis (TRANSFAC database). In addition, purine-rich elements with a 5′-GGAA-3′ core on either strand were detected, which could serve as binding sites for Ets family transcription factors. The sequence of the murine TLR2 promoter has been deposited in GenBank (accession no. AF252535), and a recent publication described the inducible regulation of murine TLR2 by NF-κB and STAT5 (28). Whereas the coding regions of human and murine TLR2 share a high degree of homology (75%), sequence comparison using a ClustalW algorithm did not reveal a significant level of homology (6–10%) between the proximal promoter regions and exons I and II of mouse and human TLR2 genes. This is in sharp contrast to the strong homology (61–77%) of corresponding regions in human and murine TLR4 genes and indicates that TLR2 may be regulated differently in both species.

Indeed, differences in TLR2 expression have been observed in mice and humans. Northern blot analysis of various mouse tissues by Matsuguchi et al. (29) revealed the highest expression of TLR2 in lung and spleen followed by thymus and brain. Tlr2 expression was also observed in murine adipose tissue (30). However, no (or very low) TLR2 expression was detected in mouse blood (29). In human tissues, the strongest TLR2 expression was detected in peripheral blood leukocytes, followed by spleen and lung; no expression was detected in human thymus (26, 27). TLR2 expression in T cells was observed only in mice, not in humans (22, 29). These differential expression patterns are likely due to the differences in TLR2 genomic sequences and regulatory context in mice and humans. As indicated in Fig. 1A, a copy of exon III (psIII) is located
~4 kb upstream of the human TLR2 gene. The presence of a non-coding pseudogene indicates that, in humans, a duplicate of the coding exon III may have been placed into a different regulatory context during evolution.

**Sp1 and Sp3 bind and activate the proximal human TLR2 promoter**

Binding sites for Sp1 family members are implicated in the regulation of several macrophage-specific genes (31–34). Several GC-rich sequences and putative binding sites for Sp1 family transcription factors are present in the proximal TLR2 promoter. As shown in Fig. 4, specific binding of Sp1 and Sp3 to the proximal putative Sp1 site was observed in EMSA using THP-1 nuclear extracts as well as HeLa cell nuclear extracts (data not shown). The oligonucleotide used in EMSA also contained a putative binding site for members of the Ets family of transcription factors, which includes PU.1, an important regulator of macrophage-specific genes. However, no specific binding of recombinant PU.1 was detectable in EMSA, and the addition of PU.1 Ab in supershift experiments had no effect on the observed band pattern (data not shown). The proximal and a putative distal Sp1 site were mutated to analyze their functional significance in reporter assays. In monocytic THP-1
cells, mutation of the proximal site showed a marked decrease in reporter activity compared with the wild-type promoter (Fig. 5A), indicating that this element is required for full reporter activity. Mutation of the putative distal site had no impact on reporter gene activity. We also deleted and mutated the putative Ets/PU motif immediately upstream of the proximal Sp1 site to determine whether this element accounts for the residual activity in the \(5\)-H11002 promoter. As shown in Fig. 5B, mutation of this site had no effect on the activity of the \(-64\) promoter. Also, deletion of both Sp1 and putative Ets elements in the \(-45\) promoter resulted in a similar decrease in reporter activity as observed using the \(-64\) promoter with a mutated Sp1 site. The results indicate the presence of additional elements important for promoter activity downstream of the proximal Sp1 site. To test the ability of Sp1 and Sp3 to transactivate the TLR2 promoter, cotransfection experiments were performed in \(Drosophila\) Schneider cells, which lack endogenous expression of Sp1 family transcription factors. As the promoter also contains several putative binding sites for Ets family transcription factors, an expression plasmid coding for PU.1, which is an important regulator of macrophage-specific genes, was also tested for its ability to transactivate the TLR2 promoter in combination with Sp1 family members. A representative reporter gene analysis is

**FIGURE 2.** Detection of alternative spliced TLR2 transcripts. RT-PCR with total RNA from monocytes (four different donors), in vitro differentiated macrophages, and myeloid cell lines MonoMac-6, THP-1, and U937 (A) or freshly isolated monocytes (0 h) and monocytes cultured for 3 h, 24 h, and 7 days (B) using primers located in exon I (87S: 5'-GTGACTGCTCGAGTCAGGC-3') and exon III (751AS: 5'-TGCCATATGCACCTCTCAGG-3'). The amplified fragments were separated by agarose gel electrophoresis along with molecular mass markers and stained with ethidium bromide. The size and composition of each detected fragment is indicated.

**FIGURE 3.** Deletion analysis of the human TLR2 promoter. Each deletion mutant was transiently transfected into myeloid THP-1 cells and nonmyeloid HeLa cells as described in Materials and Methods. Luciferase activity is relative to a CMV promoter-driven positive control and values are the mean ± SD obtained from at least three independent experiments.

**FIGURE 4.** Sp1/3 binding to a proximal binding site of the human TLR2 promoter. Labeled TLR2-Sp oligonucleotide containing the putative proximal Sp1 site as well as an adjacent putative Ets site was used in EMSA with THP-1 nuclear proteins. Addition of unlabeled oligonucleotides for competition analysis or antisera against Sp-1 family transcription factors is indicated above each lane. Arrows indicate Sp1/3-containing complexes, SS indicates Ab supershifts, and * indicates unspecific complexes.

**FIGURE 5.** A: Deletion analysis of the proximal Sp1 site showing a marked decrease in reporter activity compared with the wild-type promoter. B: Mutation of the putative distal site had no impact on reporter gene activity. C: Mutation of the putative Ets/PU motif immediately upstream of the proximal Sp1 site indicated that this element is required for full reporter activity.
shown in Fig. 6. In contrast to the +24 promoter, which was minimally activated by Sp1, Sp3, or PU.1, the −220 promoter was strongly induced by Sp1, Sp3 and PU.1 alone only weakly activated the −220 promoter; however, in combination a significant induction was observed. Both Sp3 and PU.1 further increased Sp1-mediated activation of the proximal TLR2 promoter construct.

**The human TLR2 promoter is not directly induced by microbial pattern-activated NF-κB**

The regulation of pattern recognition receptors by inflammatory cytokines or bacterial products, e.g., LPS, is of particular importance for innate immune mechanisms. In mice, TLR2 is rapidly up-regulated by multiple proinflammatory stimuli, including IL-1β, TNF, GM-CSF, and LPS (35). The activity of its promoter is induced by LPS and the induction is dependent on NF-κB- and STAT5-binding sites (28). Therefore, we investigated whether the human TLR2 promoter would respond to two proinflammatory stimuli: LPS (a known ligand for TLR4) and mycoplasmal lipopeptide MALP-2 (a known ligand for TLR2/TLR6). Initially, transient transfections were performed in THP-1 cells with and without stimulation for 4 h. Neither TLR2 construct was significantly induced by any of the two stimuli, whereas a NF-κB-responsive control plasmid was markedly induced in response to LPS or MALP-2 challenge (data not shown). We repeated the same experimental setup with MonoMac-6 cells, which secrete comparable cytokine levels upon activation as primary monocytes. As shown in Fig. 7, the NF-κB-inducible endothelial-leukocyte adhesion molecule promoter was markedly induced in response to LPS or MALP-2 challenge. However, neither the full-length nor the −220 construct was activated by either stimulus (Fig. 7). In Northern blot analyses, endogenous TLR2 mRNA levels were also not affected by LPS treatment of human THP-1 or MonoMac-6 cell lines (data not shown). Published data on TLR2 expression in LPS-stimulated human monocytes are conflicting. Some authors claimed that LPS induces TLR2 expression in human monocytes (21, 36), whereas others showed that TLR2 is not induced (22). To reinvestigate this issue, Northern blot analysis was performed on total RNA from freshly isolated monocytes and in vitro differentiated macrophages treated or untreated with LPS or MALP-2. As shown in Fig. 8, TLR2 expression was up-regulated in monocytes after 3 h of adherence in the presence of serum. No additional induction of human TLR2 was observed in LPS- or MALP-2-treated monocytes. During the differentiation of untreated cells, TLR2 expression was down-regulated after 24 h. In stimulated monocytes, higher levels of TLR2 expression were sustained. Little or no induction of TLR2 mRNA was observed in LPS- or MALP-2-treated adherent monocyte-derived macrophages (Fig. 8B). Our observations are in line with the seemingly conflicting published data. Muzio et al. (22) compared monocytes treated or untreated with LPS after 3 h and also observed no induction of TLR2 mRNA by LPS treatment. Yang et al. (21) detected an induction of TLR2 mRNA after 16 h, which probably corresponds to the differences we observed after 24 h. Finally, Visintin et al. (36) were detecting an increase of TLR2 mRNA after LPS treatment compared with freshly isolated monocytes; however, they failed to compare the induced mRNA levels with untreated adherent monocytes.
In normal tissues, CpG methylation is not involved in tissue-specific TLR2 promoter activity

Sequence analysis of the human TLR2 gene revealed that the proximal promoter, exon I, and portions of intron I are located within a CpG island (see Fig. 9). Methylation of CpG motifs has been described as a repression mechanism active in X chromosome inactivation, genomic imprinting, and silencing of mobile elements and has also been implicated in tissue-specific repression of genes (37). To investigate a possible effect of CpG methylation in the tissue-restricted expression of human TLR2, the methylation status of the proximal TLR2 promoter was analyzed by bisulfite sequencing. DNA from various cell lines, primary cell types, and different tissues was amplified and sequenced after bisulfate treatment. Whereas the promoter was unmethylated in normal tissues and primary cell types regardless of the transcriptional activity of the gene, almost complete CpG methylation was detected in several tumor cell lines and correlated with undetectable TLR2 expression. In tumors, e.g., leukemia cells such as U937, CpG methylation of the proximal promoter may be involved in down-regulation of TLR2 expression.

Discussion

In this study we investigated the transcriptional regulation of TLR2 in human monocytes/macrophages. We determined the full-length sequence and transcriptional start sites of human TLR2 and identified alternatively spliced forms of human TLR2 mRNA in human monocytes. Furthermore, we performed an initial characterization of trans-acting factors controlling the expression of human TLR2 and defined a minimal proximal promoter that confers maximal reporter activity in human monocytic THP-1 cells. We also show that human and murine TLR2 genes are regulated by structurally different promoters, which may explain the different expression patterns observed in both species.

As its murine homolog, the human TLR2 gene is composed of three exons. The first and second exons are noncoding, and the complete open reading frame is located on exon III. Alternative splicing of exon II was primarily detected in human monocytes, generating up to five different mRNA species. The shortest splicing form completely lacked exon II. The other four variants contained exon II, although it was spliced at different acceptor and donor sites. Interestingly, the relative abundance of TLR2 splicing forms varied among individuals tested, and the short form of TLR2 was detectable only in freshly isolated blood monocytes. Adjacent of monocytes to tissue culture plates rapidly and selectively induces transcription of the long splice variant. In theory, alternative splicing does not change the putative open reading frame; all mRNA isoforms are predicted to encode identical protein products. However, it is possible that differences in the 5' untranslated sequences influence the stability of the mRNA transcript or mRNA secondary structure (e.g., hairpin loop formation), which may in turn affect the extent of TLR2 protein translation. Further investigations will be needed to clarify these issues.

The 5' regulatory region of TLR2 is contained in a CpG island. Similar to many housekeeping genes and several tissue-restricted genes, the proximal TLR2 promoter was completely unmethylated in normal tissues, which excludes methylation of promoter CpGs as a mechanism for regulating TLR2 expression in normal cells. However, CpG methylation of the proximal promoter region was detected in several tumor cell lines and correlated with undetectable TLR2 expression. Interestingly, TLR2 agonists have been shown to deliver a proapoptotic signal (38, 39). Therefore, transcriptional inactivation of TLR2 expression by aberrant methylation of the TLR2 promoter may represent an advantage for tumor cell survival. Further investigations shall clarify a role of TLR2 promoter methylation in growth and survival of tumors (e.g., leukemias).

Expression of human TLR2 transcripts has primarily been detected in myeloid cells (monocytes, macrophages, dendritic cells, and granulocytes) (21, 22). The 5' proximal region of the human TLR2 gene lacks a TATA box and instead contains several GC-rich regions, which may be involved in transcriptional initiation. In reporter assays, the TLR2 promoter was strongly active in THP-1 cells but also had significant activity in HeLa cells, which do not express endogenous TLR2 message. This indicates that additional regulatory elements are involved in the observed myeloid-specific regulation of human TLR2. Our data suggest that Sp1 family transcription factors play an important role in the activation of the proximal TLR2 promoter. Although ubiquitously expressed, Sp1 family transcription factors have been implicated in the regulation of several myeloid-specific genes (31–34), most likely in collaboration with more tissue-restricted transcription factors. For example, the transcription factor PU.1 was shown to be required for the optimal expression of a growing number of myeloid-specific genes. Accordingly, PU.1 was able to collaborate with Sp3 and to
a lesser extent with Sp1 to transactivate the TLR2 promoter in Drosophila Schneider cells. Although the exact binding sites for PU.1 still need to be determined, these initial experiments suggest that PU.1 also participates in the transcriptional regulation of TLR2 in humans.

The sequence of the murine TLR2 promoter has recently been published by two groups. In contrast to human and murine TLR4, which share homologous promoter regions, the promoters of human and murine TLR2 were not conserved during evolution. We were unable to detect a significant level of homology of human and murine 5' upstream sequences or 5' untranslated regions, indicating that the coding third exon has been placed into a different genetic context in mice and humans. The different regulatory sequences provide the most likely explanation for the observed differences in TLR2 tissue distribution, e.g., T cell expression of TLR2 in mice but not in humans.

The different regulation of TLR2 in myeloid cells of both species may be of particular importance. In mice, TLR2 expression is low or undetectable in blood cells and is strongly induced by proinflammatory cytokines or microbial patterns (e.g., LPS). Accordingly, binding sites for NF-κB and STAT5 in the murine TLR2 promoter have been implicated in the rapid induction of TLR2 expression by proinflammatory stimuli in murine macrophages. However, in humans, the highest constitutive levels of TLR2 expression have been observed in peripheral blood leukocytes. In human monocytes, TLR2 expression is further up-regulated after adherence to tissue culture plates, but no additional induction was observed after short-term stimulation with bacterial products LPS or MALP-2. TLR2 expression is down-regulated in adherent monocytes after 24 h of culture, whereas monocytes stimulated with LPS or MALP-2 appear to express higher levels of TLR2. Activation of purified human blood monocytes with TLR agonists is known to inhibit their differentiation into mature macrophages, which is normally accompanied by down-regulation of TLR2 expression. This presumably leads to sustained higher levels of TLR2 mRNA in activated monocytes after 24 h of culture. In addition, marginal to no induction of TLR2 was observed in monocyte-derived macrophages. This is in sharp contrast to the high-level TLR2 induction in stimulated mouse macrophages (35).

In conclusion, our observations suggest that the human TLR2 gene is regulated by Sp1 family members, probably in collaboration with the Ets family transcription factor PU.1. Additional elements (e.g., tissue-specific enhancers and silencers) are likely to contribute to the regulation of TLR2 and are the subject of further studies. In addition, our study identifies fundamental differences in the transcriptional regulation of TLR2 in mice and humans. The observed differences in basal cell type-specific and -induced expression of TLR2 may significantly influence the immune response of both species to bacterial challenges.

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References


