PU.1 and Interferon Consensus Sequence-binding Protein Regulate the Myeloid Expression of the Human Toll-like Receptor 4 Gene*

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The protein product of the Toll-like receptor (TLR) 4 gene has been implicated in the signal transduction events induced by lipopolysaccharide (LPS). In mice, destructive mutations of Tlr4 impede the normal response to LPS and cause a high susceptibility to Gram-negative infection. Expression of TLR4 mRNA in humans is restricted to a small number of cell types, including LPS-responsive myeloid cells, B-cells, and endothelial cells. To investigate the molecular basis for TLR4 expression in cells of myeloid origin, we cloned the human TLR4 gene and analyzed its putative 5’-proximal promoter. In transient transfections a region of only 75 base pairs upstream of the major transcription initiation site was sufficient to induce maximal luciferase activity in THP-1 cells. The sequence of this region is similar in human and mouse TLR4 genes and lacks a TATA box, typical Sp1-sites or CCAAT box sequences. Instead, it contains consensus-binding sites for Ets family transcription factors, octamer-binding factors, and a composite interferon response factor/Ets motif. The activity of the promoter in macrophages was strictly dependent on the integrity of both half sites of the composite interferon response factor/Ets motif, which was constitutively bound by the myeloid and B-cell-specific transcription factor PU.1 and interferon consensus sequence-binding protein. These results indicate that the two tissue-restricted transcription factors PU.1 and interferon consensus sequence-binding protein participate in the basal regulation of human TLR4 in myeloid cells. Cloning of the human TLR4 gene provides a basis for further investigation of the possible impact of genetic variations on the susceptibility to infection and sepsis.

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‡ The abbreviations used are: LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; IC50, interferon consensus sequence-binding protein; IRF, interferon response factor; IP, PU.1 interaction partner; TLR, toll-like receptor; IL, interleukin; bp, base pair(s); IFN, interferon; PCR, polymerase chain reaction; RT, reverse transcriptase; kb, kilobase(s).

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roids (29), augmentation of the response by various bacterial infections (30–33) or by interferons (34–39), and tolerance to LPS elicited by pretreatment with LPS itself (40). TLR4 thus has become a focus of inquiry aimed at understanding each of these phenomena.

The transcriptional regulation of TLR4 is a starting point for such an inquiry. Expression of human TLR4 is restricted to a small number of cell types, including endothelial cells, B-cells, and predominantly myeloid cells (monocytes, macrophages, dendritic cells, and granulocytes) (15, 16, 21, 41). Both the basal level of TLR4 expression and its regulation in myeloid cells may influence responses to LPS and, hence, Gram-negative infection. Accordingly, we have sought to define the TLR4 promoter and to analyze those factors that govern TLR4 gene expression in human macrophages, which are the main LPS-responsive cell type. This analysis has entailed determination of the complete sequence of TLR4 and flanking genomic DNA.

EXPERIMENTAL PROCEDURES

Chemicals—All chemical reagents were purchased from Sigma-Aldrich unless otherwise noted. Protease inhibitors are from Roche Molecular Biochemicals. Oligonucleotides were synthesized by TIB Molbiol (Berlin, Germany). Antisera for supershift analyses were purchased from Santa Cruz.

Cells—Peripheral blood mononuclear cells were separated by leukopheresis of healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque. Monocytes were isolated from mononuclear cells by countercurrent centrifugal elutriation in a J6M-E centrifuge (Beckman, Munich, Germany) as described previously (42). Monocytes were >90% pure as determined by morphology and expression of CD14.

The mouse TLR4 gene was identified by hybridization screening in BAC 110p15 (Genome Systems human BAC library). Gene Cloning—The human TLR4 gene was cloned by hybridization screening in BAC clone containing the human TLR4 gene using the Expand High Fidelity PCR system (Roche Biochemicals) and the primers 5′-CTC CAT GCC ACA TCC TTC CTG AGT AAA CCT GGA GCC-3′ (sense) and 5′-CAC GCA GGA GAG GAA GCC CAT GCC TG-3′ (antisense). PCR Preparation and RT-PCR Analysis—Total RNA was isolated from different cell types by the guanidine thiocyanate/acid phenol method (44). 2.0 μg of total RNA from either cell type was reverse transcribed using oligo(dT) primer and Superscript II (Life Technologies, Inc.). Primer positions and sizes for the amplified fragments of the proximal promoter and to analyze those factors that govern TLR4 gene expression in human macrophages, which are the main LPS-responsive cell type. This analysis has entailed determination of the complete sequence of TLR4 and flanking genomic DNA.

RESULTS

Cloning and Characterization of the Human TLR4 Gene—A BAC containing the human TLR4 gene was isolated by hybridization screening, shotgun cloned and sequenced as described under “Experimental Procedures.” The mouse Tlr4 gene was similarly sequenced from a previously described BAC (43). A sequence 19 kb in length containing the human gene and a sequence 91.7 kb in length containing the mouse gene have by Calzone et al. (45). Briefly, 1 and 4 μg of poly(A) RNA were annealed to the 32P end-labeled oligonucleotide 5′-GTT GTC TTC TCT TCG AGC-3′ at 58 °C for 20 min and then cooled for 10 min at room temperature. The reverse transcription was performed with AMV RT (Promega) at 41 °C for 30 min and then stopped by addition of 8 μl of loading buffer. The amplified products were run on polyacrylamide/8 M urea sequencing gel along with sequencing products of the corresponding region of the TLR4 gene obtained with the above oligonucleotide Plasmid Construction and Purification—PCR fragments were inserted into the plasmid vector pCR2.1-TOPO (TOPO Cloning Kit, Invitrogen) for sequencing and subcloning purposes. The 4.3-kb genomic PCR fragment of the hTLR4 proximal promoter was subcloned into the insertion site of pGL3-B (Promega) and sequenced. Deletions of this construct were generated by digestion with either KpnI, Apal, HindIII, EcoRI, PsI, or XhoI and subsequent religation of the remaining plasmid. Mutations of the PU.1, OCT, and IRF-binding sites were done by PCR-mediated mutagenesis. PCR fragments with correctly introduced mutations were subcloned back into the TLR4 plasmid. A 1.2 kb of Pu.1 expression plasmid pCEC-Pu.1 was a gift from Dr. R. Maki. The expression plasmid for human ICSBP (ICSBP-pTarget) was a gift from Dr. B. Levi. The open reading frame of Pu.1 was subcloned into pGEM3 (Promega) for in vitro translation with the TNT Coupled Reticulocyte Lyase System (Promega). For transient transfections, plasmids were isolated and purified using the QiaFilter Plasmid Midi Kit from Qiagen.

Sequence Analysis—The cDNA sequencing was done by Dye Deoxy Terminator Cycle Sequencing (Applied Biosystem) according to the manufacturer's instructions, and sequences were analyzed on the Applied Biosystems DNA Sequencing System (model 373A).

Transient DNA Transfections—THP-1 cells were transfected using DEAE-dextran. 5 × 106 THP-1 cells/ml were seeded into tissue culture flasks the day before transfection. On the next day, 6 ml of cell suspension were washed twice with STBS (46) and resuspended at 2 × 106/ml in a 25 cm2 flask. The transfection mixture was removed 5 h before the addition of complete medium. 2.7 ml of reporter plasmid and 0.02 μg of Renilla luciferase control vector were mixed with DEAE-dextran (400 μg/ml) in 140 μl of STBS buffer and immediately added to the pellet THP-1 cells. The cells were incubated at 37 °C for 20 min, washed twice with STBS, resuspended, and cultured in complete RPMI medium. The two cell lines HeLa and Mel Im were transfected using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, 4 × 105 HeLa cells and 7 × 105 Mel Im cells were transfected using 5 μl of LipofectAMINE and 1 μg of total DNA (including 0.05 μg of Renilla luciferase control vector) and incubated for 5 h before the addition of complete medium. 2.7 × 105 Mel Im cells were transfected using 5 μl of LipofectAMINE and 1 μg of total DNA (including 0.05 μg of Renilla control vector) and incubated for 6 h before the addition of complete medium. A transient transfection mixture was removed after 24 h and substituted with complete medium. The transfected cell lines were cultivated for 48 h and harvested, and cell lysates were assayed for firefly and Renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega) on a Lumat LB9501 (Berthold). Firefly luciferase activity of individual transfections was normalized against Renilla luciferase activity.

Nuclear Extracts and Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared with a variation of the method of Osborne et al. (47). All buffers used contained 1 mM Na2VO4 and a mixture of protease inhibitors (2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 20 μg/ml benzamidine, 5 μg/ml E46, 50 μg/ml antipain, 100 μg/ml chymostatin). Oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The binding reaction contained 2.5 μg of nuclear extract protein reaction, 0.3 μl of the in vitro translation reaction, 0.05 μg of poly(dI-dC), 20 μM HEPES, pH 7.9, 60 mM KC1, 1 mM dithiothreitol, 1 mM EDTA, pH 8.0, 5% glycerol, and 20 nmol of probe DNA in a final volume of 10 μl. Antiseria 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 (48). Gels were fixed in 5% acetic acid, dried, and autoradiographed.
been deposited in GenBank™ (accession numbers AF177765 and AF177767, respectively). These sequences are considered to be 99.99% accurate.

Comparison of the two published cDNA sequences (accession numbers NM_003266 and U93091) for human TLR4 revealed the presence of an additional 120-bp region in the sequence reported by Rock et al. (16). A corresponding additional exon, which is not present in the mouse gene, was identified in the human TLR4 genomic sequence (Fig. 1). The presence of this exon in TLR4 transcripts introduces an in-frame stop codon, which would theoretically terminate TLR4 translation after 34 amino acids. To clarify whether this transcript is present in macrophages, we performed RT-PCR with specific primers flanking the region between exons I–IV of the human TLR4 gene. As shown in Fig. 1B, three products were amplified from macrophage and THP-1 cDNA that represent three different splicing variants of human TLR4. Sequencing of the purified fragments confirmed the specificity and nature of the three splicing forms, which is indicated in Fig. 1B. As outlined in Fig. 1C, only the transcript containing exons I, III, and IV yields the proper TLR4 protein including a putative signal peptide. The amino acid sequence as proposed by Rock et al. (16) is preceded by an in-frame stop codon and does not contain a typical leader sequence (Fig. 1C). It is therefore unlikely that the combination of exons I, II, III, and IV would yield a functional TLR protein.

To determine the transcriptional start site of the TLR4 gene we performed primer extension analysis. Two specific extension products were obtained from THP-1 RNA (Fig. 2). The major start site resides 190 bp upstream of the adenine residue of the first start codon. A second, minor initiation site was detected 74 bp upstream of the major start site.

Myeloid-specific Activity of the Proximal Human TLR4 Promoter—Published tissue Northern analyses suggest a predominant expression of hTLR4 in spleen and peripheral blood leukocytes (15, 16). RT-PCR with freshly separated and in vitro differentiated human blood cells indicated that human TLR4 is mainly expressed in myeloid cells, including monocytes, in vitro differentiated macrophages, monocyte-derived dendritic cells, granulocytes, and, to a much lesser extent, mixed lymphocytes (data not shown).

To further analyze the myeloid expression of TLR4, we cloned fragments of the 5’-proximal promoter region of the human TLR4 gene, ranging from 4.3 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 two nonmyeloid cell lines, Mel Im (melanoma) and HeLa (cervical carcinoma). Luciferase activities were normalized for transfection efficiency by co-transfection with a Renilla luciferase construct, and results for individual cell lines were compared relative to the activity of the promoter-less pGL3-basic construct. As shown in Fig. 3, the presence of both positive and negative elements can be observed. A negative regulatory region seems to reside between
nucleotides −3228 and −743. Within this region elements may exist that repress the activity of luciferase constructs specifically in THP-1 cells. Reporter constructs including residues above −385 showed low but significant reporter activity in transfected HeLa cells. However, further deletion localizes a region directing macrophage-specific reporter gene expression to approximately 75 bp proximal to the major transcriptional start site.

The Proximal Promoter Regions of Human and Mouse TLR4 Are Similar and Contain Several Purine-rich Motifs—Sequence comparison of the proximal promoter regions of both mouse and human TLR4 revealed a high degree of conservation. The 5′ proximal regions of both TLR4 genes are characterized by the absence of TATA boxes, consensus initiator sequences, or GC-rich regions found in “housekeeping” genes that normally determine transcriptional initiation (Fig. 4). They also lack Sp1 or CCAAT box sequences and instead contain several purine-rich elements with a 5′-GGAA-3′ core on either strand, a characteristic feature of many myeloid-specific genes (49–56). The smallest promoter fragment directing maximal myeloid activity (hTLR4 −75) contains two purine-rich elements that might be bound by members of the Ets family, which includes the myeloid and B-cell-specific transcription factor PU.1. In addition, a highly conserved consensus-binding site for octamer transcription factors was detected in both human and mouse proximal TLR4 promoters.

PU.1 Binds to Both Elements within the Proximal hTLR4 −75 Promoter—The importance of PU.1 recognition motifs for tissue-restricted expression of myeloid genes has been demonstrated in a growing number of cases (50–56). To test the ability of PU.1 to bind elements within the human TLR4 promoter, we performed EMSA with double-stranded oligonucleotides corresponding to the two proximal purine-rich sequences. Fig. 5A shows an EMSA with in vitro translated PU.1, which specifically bound to both oligonucleotides. In both cases, complex formation was disrupted by the addition of an excess amount of unlabeled wild type oligonucleotide but not by an oligonucleotide with a mutated 5′-GGAA-3′ core sequence. Both complexes were supershifted by the addition of a PU.1-specific antiserum. Similar experiments were done using nuclear extracts of THP-1 cells. As shown in Fig. 5B, the outer purine-rich motif (PU-11) formed a specific quickly migrating complex similar to the complex observed with in vitro translated PU.1. The complex was disrupted by the addition of a PU.1-specific antiserum but remained unchanged in the presence of antisera against two other Ets family members Fli-1 and Elf-1. The specific complex observed for the inner purine-rich motif (PU-12) was migrating significantly more slowly compared with the complex observed with in vitro translated PU.1. The complex was also supershifted in the presence of PU.1-specific antiserum but not by antisera against Fli-1 and Elf-1. The different mobility of this complex suggested the presence of at least one additional factor that interacts with PU.1 on this site.

Identification of the PU.1 Interacting Factor at the Inner

Fig. 2. Determination of the transcriptional start sites by primer extension. An oligonucleotide complementary to the 5′-end of hTLR4 cDNA was 32P end-labeled and hybridized to THP-1 mRNA. Extension products were separated on a 6% sequencing gel along with sequencing reactions primed with the identical product. The positions of the two products are marked.

Fig. 3. Deletion analysis of the human TLR4 promoter. Each deletion construct was transiently transfected into myeloid THP-1 and nonmyeloid cell lines HeLa and Mel Im as described under “Experimental Procedures.” Luciferase activity is relative to the empty control vector pGL3-B, and values are the means ± S.D. obtained from at least three independent experiments.
Purine-rich Motif—In gel shift assays nuclear extracts of various cell types, the slower migrating, PU.1 containing complex was only observed in THP.1 cells and human macrophages. In contrast to THP-1 cells, PU.1 was detectable as both slower and faster migrating band in human macrophages (Fig. 6A). To further investigate the nature of the slower migrating, PU.1 containing complex, we prepared THP-1 nuclear extracts either without protease inhibitors or without the phosphatase inhibitor Na3VO4 and performed EMSA with the PU-12 oligonucleotide. Extract preparation without protease inhibitors yielded a decrease in complex formation. Without the phosphatase inhibitor Na3VO4, the slower migrating complex disintegrated and a faster migrating complex containing PU.1 alone appeared, suggesting that phosphorylation of either factor or both is necessary for complex formation.

Further sequence analysis revealed a possible binding site for members of the family of IRF located next to the inner purine-rich motif PU-12. Interactions between PU.1 and members of the IRF family have been observed before (57–60). Fig. 7A compares the putative PU.1/IRF-binding sites of the human and mouse TLR4 promoter with so far identified sequences of similar binding sites in the human gp91phox (58), CD20 (59), murine IL-18 promoters (60), and immunoglobulin light chain enhancers E{l}_{L2–4} and E{k}_{3–9} (57). We next performed EMSAs with specific antisera against the three IRF family members (ICSBP, interferon-stimulated gene factor 3g, and PIP) to identify the interacting partner of PU.1 in the context of the hTLR4 promoter. As shown in Fig. 7B, a strong supershift was detected with THP-1 extracts and the ICSBP antiserum. A supershift (albeit less intense) was also observed with the PIP antiserum, suggesting the presence of PIP, which is generally thought to be lymphocyte-specific (57), or a PIP cross-reactive protein in THP-1 nuclear proteins. No supershift was detectable with the interferon-stimulated gene factor 3g antiserum.

In vitro translated ICSBP was unable to bind the PU-12 element independently but was able to interact with PU.1 to form a complex with slightly decreased mobility to that observed in THP-1 nuclear extracts (Fig. 7C). The complex of in vitro translated PU.1 and ICSBP was supershifted with the ICSBP and the PIP antiserum, indicating that the latter is cross-reactive with human ICSBP protein in gel shift assays. These results suggested that ICSBP and PU.1 are the main components of the slower migrating PU-12 complex. A similar band pattern of ICSBPPU.1 and predominantly PipPU.1 containing complexes was observed in gel shift experiments performed with nuclear extracts from human B cells, which also express PU.1 and both PIP and ICSBP (data not shown). Sequence comparison revealed a single base pair difference in the IRF-binding site between mouse and human promoter (Fig. 3). Binding of the PU.1/ICSBP complex was also detectable using the corresponding mouse sequence (mPU-12; Table I) and THP-1 nuclear extracts, although the band corresponding to PU.1 binding...
ing alone increased relative to the human sequence (data not shown).

A Conserved Element Adjacent to the Composite ICSBP-PU.1 Motif Is a Weak Binding Site for Octamer Transcription Factors—A highly conserved octamer element is present in both human and mouse TLR4 promoters, which prompted us to investigate its interaction with POU domain transcription factors. Direct binding of nuclear proteins to the site was almost undetectable in THP-1 cells and macrophages (data not shown); however, competition studies with macrophage nuclear proteins and the consensus octamer motif (OCT consensus) identified the TLR4 octamer element as a weak binding site for either half-side of the PU.1/ICSBP motif showed a markedly reduced activity compared with the wild type promoter (Fig. 9). The effect of the PU.1 mutation was most distinct, and reporter activity was almost completely abolished. In contrast, disruption of the octamer-binding site showed a trend toward a slightly increased activity, although statistical analysis revealed that the difference was not significant. Mutation of the outer PU.1 motif also had no significant impact on the activity of the hTLR4 promoter (Fig. 9).

To analyze the functional significance of the four identified binding sites in reporter assays, we used site-directed mutagenesis to abolish each of the four putative binding sequences. The oligonucleotides carrying the mutated binding sites (PU-11M, PU-12M, IRF-M, and OCT-M; for sequences see Table I) did not compete with complex formation of wild type oligonucleotides in gel shift assays (data not shown). In monocytic THP-1 cells the reporter constructs with mutations of either half-side of the PU.1/ICSBP motif showed a markedly reduced activity compared with the wild type promoter (Fig. 9). The effect of the PU.1 mutation was most distinct, and reporter activity was almost completely abolished. In contrast, disruption of the octamer-binding site showed a trend toward a slightly increased activity, although statistical analysis revealed that the difference was not significant. Mutation of the outer PU.1 motif also had no significant impact on the activity of the hTLR4 promoter (Fig. 9).

FIG. 5. PU.1 binding to GGAA motifs within the −75 hTLR4 promoter. Labeled PU-11 and PU-12 oligonucleotides were used in EMSA with in vitro translated PU.1 protein (A) or THP-1 nuclear proteins (B). Addition of unlabeled oligonucleotides for competition analysis or antisera against Ets family transcription factors are indicated above each lane. PU.1 containing complexes are marked with arrows, and antibody supershifts are marked with SS, and unspecific complexes are marked with asterisks.

FIG. 6. Specificity and stability of the slower migrating complex. A, EMSA using labeled PU-12 oligonucleotide and nuclear extract preparations of various cell types. B, THP-1 nuclear extracts were prepared either without protease inhibitors or without the phosphatase inhibitor Na3VO4, as indicated above each lane. EMSA is shown for the labeled PU-12 oligonucleotide. PU.1 containing complexes are marked with arrows, and unspecific complexes are marked with asterisks.

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Mutational Analysis of the Identified Binding Sites and Effect of IFN-γ—To analyze the functional significance of the four identified binding sites in reporter assays, we used site-directed mutagenesis to abolish each of the four putative binding sequences. The oligonucleotides carrying the mutated binding sites (PU-11M, PU-12M, IRF-M, and OCT-M; for sequences see Table I) did not compete with complex formation of wild type oligonucleotides in gel shift assays (data not shown). In monocytic THP-1 cells the reporter constructs with mutations of either half-side of the PU.1/ICSBP motif showed a markedly reduced activity compared with the wild type promoter (Fig. 9). The effect of the PU.1 mutation was most distinct, and reporter activity was almost completely abolished. In contrast, disruption of the octamer-binding site showed a trend toward a slightly increased activity, although statistical analysis revealed that the difference was not significant. Mutation of the outer PU.1 motif also had no significant impact on the activity of the hTLR4 −75 promoter (Fig. 9).

To investigate whether the activity of the TLR4 promoter could be induced in a nonmyeloid cell line that does not express either PU.1 or ICSBP, we co-transfected HeLa (cervical carcinoma) cells with the hTLR4 promoter and expression plasmids for PU.1 (PU-pECE) and/or ICSBP (hICSBP-pTarget). We only observed a slight up-regulation of relative luciferase activity (1.5-fold) when PU.1 was co-transfected alone (data not shown). Co-transfection of PU.1 and ICSBP expression plasmids had no cooperative effect on the promoter activity in HeLa cells with or without the addition of IFN-γ. Similar results have been reported for the functional ICSBP-PU.1-binding motif of the CYBB gene, which encodes for gp91phas (58). This might indicate that additional factors, which are not present in HeLa cells, are required for the induction of the TLR4 promoter by PU.1/ICSBP or that inhibitory proteins are present that prevent PU.1/ICSBP binding and transactivation.

IFN-γ, a cytokine released by activated T lymphocytes, is a major activator of macrophage microbicidal functions and enhances both sensitivity and magnitude of their response to LPS (36, 37, 39, 40, 61). In mouse macrophages, priming with IFN-γ drastically up-regulates expression of the transcription factor ICSBP (62), which has been implicated in IFN-γ-mediated gene regulation (63–65). These observations prompted us to investigate a possible effect of IFN-γ on PU.1/ICSBP complex formation in macrophages. Constitutive binding of the ICSBP-PU.1 complex observed in untreated cells was only slightly increased during IFN-γ stimulation for 3, 6, or 18 h. No additional bands
were observed upon IFN-γ treatment, and the ICSBP-PU.1 complex was completely supershifted by the ICSBP antiserum (data not shown). Whereas high levels of IRF-1 transcripts were detectable after IFN-γ treatment, suggesting efficient IFN-γ priming (66), only a slight induction (approximately 2-fold) of all three TLR4 splicing forms by IFN-γ treatment was detectable by RT-PCR using total RNA from in vitro differentiated macrophages or THP-1 cells (data not shown). IFN-γ treatment of THP-1 cells did not affect the intensity of the ICSBP-PU.1-DNA complex in gel shift assays, and the relative activities of TLR4 promoter constructs in transient transfections of THP-1 cells were not affected by INF-γ treatment either 24 or 4 h before cell harvest (data not shown).

DISCUSSION

In this study we investigated the transcriptional regulation of TLR4 in human macrophages, the main LPS-responsive cell type. We cloned and sequenced 19 kb of the human TLR4 gene, sequenced 91.7 kb of the mouse Tlr4 gene, determined the transcriptional start sites, and identified three alternative splicing forms of human TLR4 in human myeloid cells. Furthermore, we performed an initial characterization of regulatory elements controlling the expression of human TLR4 and defined a minimal proximal promoter that confers full reporter activity in human monocytic THP-1 cells.

Gene cloning and sequence comparison revealed the existence of an additional exon (exon II) in the human TLR4 gene, which is not present in the mouse gene. Its integration into TLR4 mRNA introduces an in-frame stop codon, which prompted us to investigate the presence and ration of alternative splicing forms of TLR4. By RT-PCR, we were able to detect three alternative splicing variants of hTLR4; however, only one of them encodes the proper TLR4 protein. Although we did not observe major changes in the relative abundance of the three transcripts in myeloid cells, alternative splicing could represent an additional level of TLR4 regulation in humans. Disruptions of alternative splicing have been correlated with some human genetic diseases (67); mutations that affect the splicing of TLR4 mRNA could favor the expression of noncoding transcripts. It is also possible that alternative splicing events are regulated in response to developmental or physiological cues (67). As a consequence, the expression of TLR4 protein could, for example, be switched off, if the second exon is included in all transcripts. However, further investigations will be needed to clarify these issues.

Expression of human TLR4 transcripts has primarily been detected in myeloid cells (monocytes, macrophages, and dendritic cells) and some B-cell lines (15, 16, 27). In accordance with the observed cell type restricted expression, the TLR4 gene is controlled by a typical myeloid type promoter in macrophages. The 5′-proximal region lacks a TATA box, consensus initiator sequences, or GC-rich regions found in “housekeeping” genes and instead contains multiple purine-rich sequence motifs that are recognized by transcription factors of the Ets family, including PU.1. The transcription factor PU.1 was
shown to be required for the optimal expression of a growing number of myeloid-specific genes (49–56, 68–70). Our data suggest that PU.1 also plays an important role in the myeloid expression of TLR4. In human macrophages, PU.1 binds an essential motif in the proximal promoter and recruits ICSBP to an adjacent binding site that is also required for the optimal activity of the promoter.

ICSBP is a member of the IRF family of transcription factors and is expressed mainly in cells of hematopoietic origin (71). Its mouse homologue was originally cloned as interferon-γ-regulated protein that bound an interferon-inducible enhancer element of major histocompatibility complex class I genes (71). Because of the phosphorylation of tyrosine residues in vivo, ICSBP does not bind to DNA by itself but has been shown to interact with two other IRF family members (IRF-1 and IRF-2) (72, 73). Cooperative DNA binding of ICSBP with one of the other IRF family members results in an increased binding activity for IFN-stimulated response elements and transcriptional repression of genes containing the IFN-stimulated response elements (57, 63–65, 74). In addition to its interaction with members of its own gene family, ICSBP, and the related lymphoid-restricted factor Pip (ICSAT/IRF-4) are able to form complexes with the Ets family member PU.1 (57). ICSBP or Pip protein bind a composite IRF/Ets motif only in the presence of PU.1, and complex formation requires the phosphorylation of PU.1 at serine 148 (57, 75). Composite IRF/Ets-binding sites have been implicated in cell-type-specific gene expression in myeloid and B-cells. The CD20 promoter and immunoglobulin light chain enhancers E<sub>B</sub> and E<sub>E</sub> contain IRF/Ets-binding sites that seem to be important for their B-cell-specific regulation and are bound by PU.1 and Pip (57, 59). An IRF/Ets motif, which is bound by PU.1 and ICSBP, seems important for the myeloid-restricted expression of gp91<sub>p72</sub> (58). Kim et al. (60) recently described an ICSBP-binding site that was critical for the activity of the murine 5′-flanking IL-18 promoter. Although ICSBP usually does not bind DNA alone, the authors did not investigate the presence of additional factors in the observed ICSBP-DNA complex. The binding sequence of the IL-18 promoter is similar to other IRF/Ets motifs (Fig. 7A); it is therefore likely that Ets factors (most likely PU.1) also participate in the regulation of the 5′-flanking IL-18 promoter.

We identified a similar site in the human TLR4 promoter and showed that both PU.1 and ICSBP bind to adjacent elements that are indispensable for the full activity of the TLR4 promoter. As expected, formation of the ternary complex is dependent on the presence of PU.1 and its state of phosphorylation. These data suggest that the interaction between the two tissue-specific transcription factors PU.1 and ICSBP is important for the basal activity of the TLR4 promoter in human myeloid cells. Preliminary gel shift analyses using B-cell extracts indicate the presence of a similar complex. The composite IRF/Ets motif may therefore also contribute to the expression of TLR4 in human B-cells. Using in vitro translated proteins, we observed that the complex between PU.1 and ICSBP migrates slightly faster than the native THP-1 complex. This difference could be due to altered post-translational modifications or an effect of the (5–10-fold) higher total protein concentrations in the preparations of in vitro translated proteins. However, it is also possible that the native THP-1 complex contains another, as yet unidentified protein.

We identified constitutive nuclear ICSBP protein in the human monocytic cell line THP-1 and in vitro differentiated human macrophages, which is consistent with the previously described constitutive expression of ICSBP in the human pre-monocytic cell line U937. This indicates another more constitutive expression in human macrophages rather than a strictly inducible expression pattern, which is described for mouse macrophages, where constitutive expression of ICSBP is undetectable, and both mRNA and protein levels are strongly induced after stimulation with IFN-γ (62). If at all, ICSBP and the IRF/Ets motif in the TLR4 promoter only weakly confer IFN-γ responsiveness. The ICSBP-PU.1 complex was slightly induced by IFN-γ in human macrophages, and in both THP-1 cells and human macrophages, TLR4 mRNA expression was slightly enhanced by IFN-γ priming. However, IFN-γ stimulation did not significantly affect the activity of TLR4 promoter constructs in THP-1 cells. It remains to be clarified whether the observed increase in TLR4 message is regulated on the level of gene transcription or whether other mechanisms (e.g. mRNA stability) mediate the IFN-γ induced increase in TLR4 mRNA.

ICSBP-deficient mice show altered antiviral and antibacterial responses and develop a chronic myelogenous leukemia-like syndrome (76). An observed failure to develop Th-1-driven immune responses has been correlated with a defect in IL-12 p40 production by cells of myeloid origin (26, 77). The response of ICSBP-deficient mice to LPS is described as normal (77). However, it is not clear whether the murine Tlr4 gene is similarly dependent on the IRF/Ets motif, which seems to form a less stable complex with ICSBP and PU.1. Analysis of the mouse promoter will clarify whether ICSBP plays a role in the transcriptional regulation of Tlr4 in mice.

In conclusion, our observations suggest that a functional cooperation between PU.1 and ICSBP regulates the myeloid
expression of TLR4 in humans. Additional elements and mechanisms are likely to contribute to the regulation of TLR4 in health and disease and are the subject to further studies. Transcriptional regulation, alternative splicing events, or function of TLR4 protein could be altered because of mutations or polymorphisms of the TLR4 gene. This study provides the basis for further investigating a possible correlation of yet unknown genetic variations with a higher susceptibility for infection and septic complications.

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