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Cutting Edge: Naturally Occurring Soluble Form of Mouse Toll-Like Receptor 4 Inhibits Lipopolysaccharide Signaling¹

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Toll-like receptors (TLRs) are a family of proteins playing important roles in host defense. Mice defective of functional TLR4 are hyporesponsive to LPS, suggesting that TLR4 is essential for LPS signaling. Here we report the cloning of an alternatively spliced mouse TLR4 (mTLR4) mRNA. The additional exon exists between the second and third exon of the reported *mTLR4* gene and contains an in-frame stop codon. The alternatively spliced mRNA encodes 86 aa of the reported mTLR4 and an additional 36 aa. This alternatively spliced mTLR4 mRNA expressed a partially secretory 20-kDa protein, which we named soluble mTLR4 (smTLR4). In a mouse macrophage cell line, the exogenously expressed smTLR4 significantly inhibited LPS-mediated TNF- α production and NF- κ B activation. Additionally, in mouse macrophages, LPS increased the mRNA for smTLR4. Taken together, our results indicate that smTLR4 may function as a feedback mechanism to inhibit the excessive LPS responses in mouse macrophages. *The Journal of Immunology*, 2001, 165: 6682–6686.

Gram-negative bacteria represent a major group of pathogens causing serious infection. A glycolipid known as endotoxin or LPS is the principal bacterial constituent recognized by the innate immune system. LPS stimulates host cells such as monocytes, macrophages, and B cells through the activation of transcription factors such as NF- κ B (1, 2). Also, recent reports including ours have indicated that a fraction of T cells respond to LPS by acquiring Th1-like phenotypes (3–5).

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Toll, first identified in *Drosophila* (6), participates in antimicrobial immune responses (7). Several mammalian Toll homologs have recently been identified (8). In addition to their cytoplasmic domains homologous to IL-1 receptor family proteins (6), they also share leucine-rich repeats (LRRs)³ in the extracellular region. One of the mammalian Toll homologs, Toll-like receptor (TLR)2, was shown to function as an LPS signaling receptor (5, 9, 10). However, more recent studies found *TLR4* gene in the chromosomal region responsible for the defective LPS responses in some mouse strains (*lps*) (11, 12). These studies also found independent mutations in the *TLR4* genes of two LPS-hyporesponsive mouse strains (C3H/HeJ and C57BL/10/ScCr), strongly suggesting that TLR4 is the dominant receptor for LPS in vivo.

In our current study, we report the molecular cloning of an alternatively spliced mouse TLR4 (mTLR4) mRNA form, encoding 122 aa. When expressed in Chinese hamster ovary (CHO)-K1 cells, this protein was partly secreted into the culture medium, thus named soluble mTLR4 (smTLR4). Exogenous expression of smTLR4 in a mouse macrophage cell line inhibited LPS-mediated TNF- α production and NF- κ B activation. Additionally, LPS stimulation increased the smTLR4 mRNA level, suggesting that smTLR4 might be induced as a feedback mechanism to inhibit the excessive LPS responses in mouse macrophages.

Materials and Methods

Reagents and cells

LPS from *Escherichia coli* serotype B6:026, FCS, and the anti-Flag M2 mAb were obtained from Sigma (St. Louis, MO). A mouse T cell line, EL-4, and CHO-K1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A mouse macrophage cell line, RAW264.7, was obtained from RIKEN cell bank (Tsukuba, Japan).

Isolation of the full-length cDNA clone encoding smTLR4

In an effort to clone the mTLR4 cDNA, a 500-bp DNA probe was generated by RT-PCR corresponding to the 5' region of published TLR4 DNA starting from the translational initiation site using the total RNA from a mouse macrophage cell line, J774.1, as a template. The synthesized cDNA fragment was ³²P-labeled by random priming and used for screening a J774.1 cDNA library constructed in the Uni-Zap cloning vector (Stratagene, La Jolla, CA), provided by Dr. H. Yagita (Juntendo University, Tokyo, Japan). Plaque hybridization was conducted as previously described (5). The inserts of the positive phage clones were excised using the

³ Abbreviations used in this paper: LRR, leucine-rich repeat; TLR, Toll-like receptor; mTLR4, mouse TLR4; smTLR4, soluble mTLR4; PI3-kinase, phosphatidylinositol 3-kinase; ORF, open reading frame; CHO, Chinese hamster ovary.

Rapid Excision Kit (Stratagene) and recloned in the pBlueScript vector (Stratagene). DNA sequence analysis was performed on these plasmids using a DNA sequencer (model 373A; Perkin-Elmer Biosystems, Foster City, CA) and a Thermo Sequence cycle sequencing kit (Perkin-Elmer Biosystems).

Expression plasmids

For the Flag-tagged smTLR4 expression plasmid, the coding region of smTLR4 was amplified by PCR from the isolated alternatively spliced mTLR4 cDNA and cloned into the *AscI* site of the expression plasmid, pEFBOS-Flag, which encodes a C-terminal Flag epitope (5).

Transient transfection

CHO-K1 or RAW cells were plated onto 35-mm plates at 1×10^6 cells/plate on the day before transfection. Combinations of expression plasmid DNAs (2 μ g total/plate) were transfected using Lipofectamine (Life Technologies, Grand Island, NY). Cells were used for further analysis at 48 h after transfection.

RT-PCR and Northern blot analysis

Total cellular RNA was prepared using TRIZOL reagent (Life Technologies). cDNA was synthesized from 2 μ g of total RNA by extension of random primers with 200 U of Superscript II (Life Technologies). PCR of the synthesized cDNA was performed as previously described (5). The synthesized PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The primers were: mouse β -actin sense, TGGAAATCCTGTGGCATCCATGAAAC; β -actin antisense, TAAAACGCAGCTCAGTAACAGTCCG; mTLR4 sense, AGTGGTCAAGGAACAGAAGCA; mTLR4 anti-sense, CTTTACCAGCTCATTCTCACC; alternatively spliced mTLR4 sense, TTATCCAGGACATGATA; anti-sense, TCACACCATTTTCCTTTA. The primers for the alternatively spliced mTLR4 are directed to the junction of the reported second exon/the alternative exon and the alternative exon/the reported third exon, respectively, and were confirmed not to synthesize PCR products from the mouse genomic DNA.

For Northern blot analysis, messenger RNA was isolated from total RNA using the mRNA Isolation Kit (Roche Molecular Biochemicals). RNA blotting was conducted as previously described (5).

Cell extract preparation and immunoblotting

Cells were lysed in RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM Na_3VO_4 , 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin) at 10^8 cells/ml. The lysates were analyzed by immunoblotting as previously described (5).

Immunoprecipitation

Supernatants were collected from CHO-K1 cells transiently transfected with pEFBOS-Flag or pEFBOS-smTLR4/Flag. smTLR4/Flag was immunoprecipitated with the anti-Flag M2 mAb and protein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) before analysis by immunoblotting.

Subcellular localization of smTLR4

CHO-K1 cells were transiently transfected with pEFBOS-smTLR4/Flag as described above. At 48 h posttransfection, the cytosolic and membrane fractions were isolated as previously described (13).

Cytokine ELISA

RAW264.7 cells transiently transfected with pEFBOS-Flag or pEFBOS-smTLR4/Flag were cultured with LPS for 24 h. Production of TNF- α in the supernatant was measured by a TNF- α ELISA system (Genzyme, Boston, MA), and the data were presented as the mean \pm SD of triplicate samples.

Luciferase assays

Raw264.7 cells were transiently transfected with 0.8 μ g of pGL3-NF- κ B/Luc (a luciferase reporter construct containing the consensus NF- κ B binding sequence), 0.2 μ g of pRL/SV40 as an internal control (Promega, Madison, WI), and either 1.0 μ g pEFBOS-Flag or pEFBOS-smTLR4/Flag. At 48 h after the transfection, cells were stimulated with LPS for 8 h, lysed, and the luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions. Background luciferase activity was subtracted, and the data were presented as the means \pm SD of triplicate samples.

Results

Molecular cloning of an alternatively spliced mTLR4 cDNA

As several laboratories have reported (28) multiple mRNA isoforms were detected for mTLR4 in the Northern blot analysis (Fig. 1A). For the purpose of identifying mTLR4 mRNA isoforms, a mouse macrophage cDNA library was screened with a 0.5-kb probe directed to the 5' region of the published mTLR4 cDNA. Three independent clones were isolated from 8×10^5 plaques and analyzed by DNA sequencing. Two of the clones exactly matched the published mTLR4 cDNA sequence, whereas the other had a 144-bp insertion between the reported second and third exon sequences (Fig. 1B). This 144-bp sequence was found in the reported mTLR4 genomic DNA (accession number AF177767) and matched the AG/GT rule suggesting that it was produced by the alternative splicing. This novel exon contains an in-frame stop codon at 110 bp (Fig. 1C).

Analysis of the predicted amino acid sequence indicates that the alternative spliced cDNA encodes a 122-aa protein. The first 86 aa are identical with those of the extracellular domain of TLR4, whereas aa 87–122 diverge. This additional region showed some homology (73% positives) with the N-terminal region of mouse phosphatidylinositol 3-kinase (PI3-kinase) (14).

Regulation of the alternatively spliced TLR4 mRNA expression in mouse macrophages and T cells

To analyze the effects of LPS on the TLR4 alternatively spliced mRNA expression, RAW264.7, a well-established mouse macrophage cell line, was treated for 1, 2, 4, or 24 h with LPS, and total RNAs were isolated. The expression of the alternatively spliced mRNA was visualized by a pair of primers that both span the junctions of the alternatively spliced exon and the published exon sequences. The total TLR4 mRNA level was measured by a pair of primers directed to the 3' region of the TLR4 cDNA common to the published TLR4 cDNA and the alternatively spliced form. In the semiquantitative RT-PCR using these two pairs of primers, LPS increased the alternatively spliced TLR4 mRNA level within 4 h (Fig. 2A). The

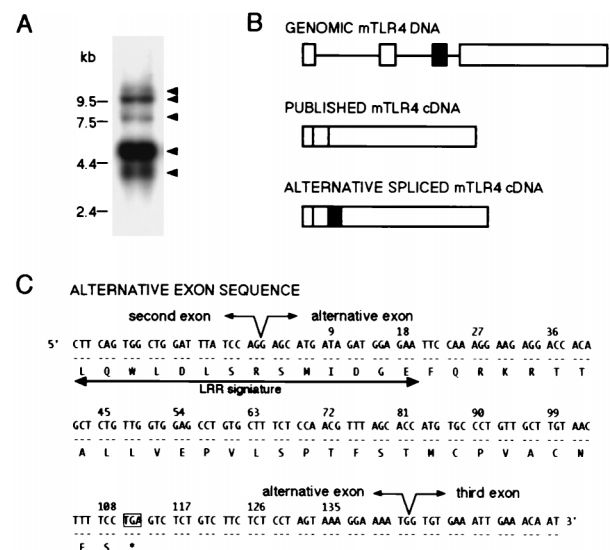


FIGURE 1. Alternative splicing of *mTLR4* gene. *A*, Northern blot analysis of mTLR4. RNA size in kilobases is depicted on the left of the figure; arrows on the right indicate mRNA species that specifically hybridized with the mTLR4 probe. *B*, Structure of the *mTLR4* gene. Exons are shown as boxes, introns as thin lines, and alternative exons as black boxes. The alternative exon is located between the reported second and third exon. *C*, Nucleotide and polypeptide sequence of the alternative exon. There is an in-frame stop codon at 110 bp (shown as a box). The 5' region of the alternatively spliced exon contributes to an additional LRR signature sequence (underlined).

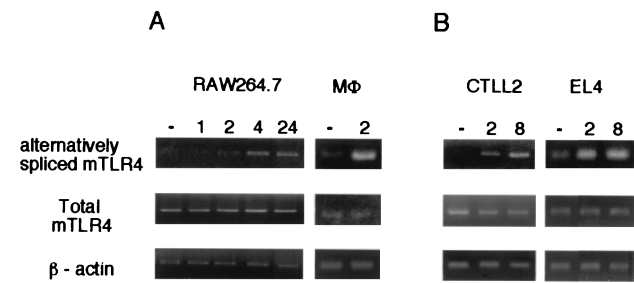


FIGURE 2. LPS and anti-CD3 treatment increased the alternatively spliced TLR4 mRNA level in macrophages and T cell lines, respectively. **A**, RAW264.7 and macrophages derived from a BALB/c mouse spleen were stimulated with LPS for indicated times or left unstimulated (–). Total RNAs were isolated, and RT-PCR analysis was performed using the indicated primers. **B**, CTLL2 and EL4 cells were stimulated with anti-CD3 Ab for indicated times or left unstimulated (–). Total RNAs were isolated and RT-PCR analysis was performed.

mRNA level remained elevated at least until 24 h after the LPS treatment. In contrast, no significant increase was observed in the total TLR4 mRNA level after LPS treatment (Fig. 2A). The similar results were also obtained with freshly isolated mouse splenic macrophages (Fig. 2A).

We have previously reported that TLR4 is constitutively expressed in freshly isolated mouse T cells (5). Thus, we also analyzed the gene expression of the alternatively spliced mTLR4 mRNA in two mouse T cell lines. Two well-established T cell lines, CTLL2 and EL4, were treated for 2 or 8 h with anti-CD3ε Ab, and total RNAs were isolated. The anti-CD3ε Ab treatment failed to increase the total TLR4 mRNA level but increased the alternatively spliced mTLR4 mRNA level in both T cell lines (Fig. 2B). Additionally, this mRNA isoform was detected in various mouse tissues including brain, heart, lung, kidney, liver, thymus, spleen, small intestine, and testis (data not shown).

The alternatively spliced TLR4 mRNA encodes a soluble 20-kDa protein

As the extra exon has a stop codon in frame, two open reading frames (ORFs) were possible in the alternatively spliced TLR4 mRNA. Thus the two putative ORFs were prepared by RT-PCR using the total RNA prepared from a mouse macrophage cell line, J774.1, as a template, and cloned in frame with the 3' Flag tag in the expression vector, pEFBOS (15). These two expression plasmids were transiently transfected into CHO-K1 cells, and protein expression was analyzed by anti-Flag Ab. ORF (3') did not express a detectable amount of protein, probably suggesting that the produced protein was unstable (data not shown). In contrast, in the cells transfected with 5' ORF expression plasmids, a 20-kDa protein was detected (Fig. 3A). This 20-kDa protein was easily detected in the culture supernatant, suggesting that it is a soluble protein and thus called smTLR4. Additionally, in the subcellular localization experiment, most of this protein was detected in the membranous compartment (Fig. 3B).

smTLR4 inhibits LPS-mediated NF-κB activation and TNF-α production in RAW264.7 cells

To determine whether smTLR4 had any physiological functions, we transfected RAW264.7 cells with the expression plasmid encoding smTLR4, pEFBOS-smTLR4/Flag. Forty-eight hours after the transfection, cells were stimulated with various concentrations of LPS for 24 h, and the culture supernatants were collected for the measurement of TNF-α. As shown in Fig. 4A, the expression of smTLR4 inhibited LPS-mediated TNF-α production from RAW264.7. We also analyzed LPS-mediated NF-κB activation in RAW264.7 cells expressing smTLR4. An NF-κB/Luc reporter plasmid was cotransfected with the smTLR4 expression plasmid into RAW264.7 cells. Forty-eight hours after the transfection, cells were stimulated with LPS for 8 h and NF-κB activation was measured by the luciferase assay. As shown in Fig. 4B, the expression of smTLR4 inhibited LPS-mediated NF-κB activation in RAW264.7 cells.

Discussion

In this study, we have described the molecular cloning of an alternatively spliced form of mTLR4 mRNA. It encodes a soluble 20-kDa protein, which we named smTLR4. LPS stimulation of

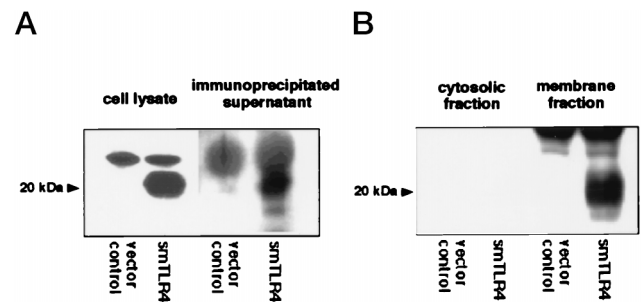


FIGURE 3. The alternatively spliced mTLR4 mRNA expressed a soluble 20-kDa protein. **A**, CHO-K1 cells were transiently transfected with pEFBOS-smTLR4/Flag or pEFBOS/Flag. At 48 h after the transfection, cell lysates and culture supernatants were prepared. The cell lysates and the anti-Flag immunoprecipitates of the culture supernatants were fractionated by SDS-PAGE for the immunoblotting with anti-Flag Ab. **B**, Subcellular localization of the smTLR4. The membrane and cytosolic fractions were prepared from the smTLR4-expressing CHO-K1 cells as described in *Materials and Methods*. The distributions of the smTLR4 were determined by Western blotting with anti-Flag Ab.

macrophages and TCR engagement of T cell lines increased the alternatively spliced mTLR4 mRNA level although the total TLR4 mRNA level remained constant. Finally, the expression of smTLR4 inhibited the LPS-mediated responses (NF-κB activation and TNF-α secretion) in a mouse macrophage cell line.

This alternatively spliced mTLR4 mRNA contains an in-frame stop codon in its additional exon. In the originally reported mTLR4 mRNA, the first, second, and most of the third exon encodes the extracellular domain (Genebank accession number AF177767). As the additional exon is located between the second and third exon, it results in the expression of a protein consisting of 86 aa of the extracellular domain of the reported mTLR4 protein and an additional 36 aa. The encoded protein has a signal peptide but lacks the obvious transmembrane and intracellular domains. Thus, it is assumed that it can be secreted. In fact, we could easily detect the protein in the culture supernatant in a transient transfection assay. However, it is of note that a significant proportion of the protein remained in the cell lysate (Fig. 3A). The size of this protein in the cells is also ~20 kDa, suggesting it is posttranslationally modified (probably glycosylated), and it was most localized in the membrane (Fig. 3B). Consistent with this, there is a stretch of hydrophobic amino acids in the C-terminal region of the protein (Fig. 1C).

The extracellular segments of TLR proteins are distinctively composed of 24–28 aa-long LRRs (8). LRRs are also found in *Drosophila* homologs, Toll, and 18 wheeler (16, 17), suggesting that LRRs are functionally essential for the Toll family proteins. Although amino acid sequence homology between human and mouse TLR4 is higher in the cytoplasmic domains, the LRR structure is also conserved well, suggesting that it plays a critical role in their biological functions. We searched for conserved alignments about smTLR4 with Block Searcher (http://blocks.fhcrc.org/blocks/blocks_search.html). Interestingly, in addition to the LRRs common to the originally reported mTLR4, the C-terminal amino acids that are not found in the originally reported sequence contribute to an additional LRR signature (Fig. 1C). Thus, it is possible that smTLR4 has functions divergent from the N-terminal region of the originally reported TLR4, such as qualitatively or quantitatively different binding affinities. Interestingly, in an amino acid sequence homology search, the additional 36 aa sequence showed homology (73% positives) with the N-terminal region of mouse PI3-kinase (14). The function of this N-terminal region of

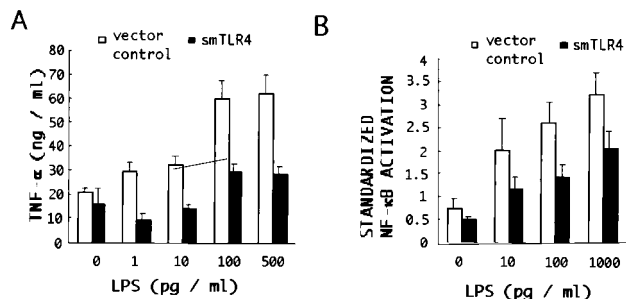


FIGURE 4. Soluble mTLR4 inhibits LPS-mediated TNF- α production and NF- κ B activation in RAW264.7 cells. *A*, RAW264.7 were transiently transfected with 2 μ g of smTLR4 expression plasmid or the empty vector. At 48 h after the transfection, cells were stimulated with various concentrations of LPS for 24 h, and TNF- α concentrations in the supernatants were measured by ELISA. *B*, RAW264.7 were transiently cotransfected with 0.8 μ g NF- κ B luciferase reporter plasmids, 0.2 μ g pRL/SV40 as an internal control, and 1.0 μ g of either the smTLR4 expression plasmid or the empty vector. At 48 h after the transfection, cells were stimulated with various concentrations of LPS for 24 h. The cells were lysed and the luciferase activities were measured.

PI3-kinase is unknown, thus the significance of this homology remains obscure.

It was noteworthy that the overexpression of smTLR4 inhibited LPS-mediated TNF- α production and NF- κ B activation in a mouse macrophage cell line, RAW264.7 (Fig. 4). Several explanations are conceivable for this inhibitory effect. The extracellular domain of TLR4 has been thought to interact with LPS and smTLR4 contains a part of it, raising a possibility that it may compete for LPS binding. However, at present, there is no strong evidence for high-affinity interaction of LPS with any members of the Toll family. CD14 is involved in mediating LPS responses by binding LPS with high affinity (18). This binding seems to require a serum factor, LPS binding protein, which is a plasma lipid transfer protein that transfers LPS from the bacterial membrane to the LPS-binding site of CD14. Thus, smTLR4 may interact with CD14 and/or LPS binding protein and inhibit LPS signaling via TLR4. In the preliminary experiments, we could not inhibit the LPS-mediated NF- κ B activation by the supernatant transfer of the smTLR4-transfected cells (data not shown). However, this does not rule out the possibility that smTLR4 exerts its inhibitory effect after secretion, because smTLR4 may be locally highly concentrated in the vicinity of the transfected cells.

It has also been reported that TLR4 alone is not capable of sensing the presence of LPS and works with a secretory molecule, MD-2, which can be coprecipitated with TLR4 (19). Treatment of cells with an Ab that recognizes TLR4-MD2 complex inhibited LPS signals, suggesting that MD2 is essential in mediating LPS responses (20). Thus, it is also possible that smTLR4 may inhibit TLR4-MD2 interaction that is necessary for the proper receptor complex.

Another possibility is that the inhibitory effect of smTLR4 occurs in the membrane. Although smTLR4 can be secreted into the culture supernatant, a significant amount of the protein was also found in the membrane compartment (Fig. 3). smTLR4 may interact with the newly synthesized TLR4 or CD14 in the membrane and block its signal transduction.

As smTLR4 inhibits LPS-mediated signals and smTLR4 mRNA is induced by LPS, it is reasonable to presume that smTLR4 works as a feedback mechanism to inhibit the excessive LPS responses of macrophages. Additionally, our results have demonstrated that smTLR4 mRNA level is also increased in T cells by TCR engage-

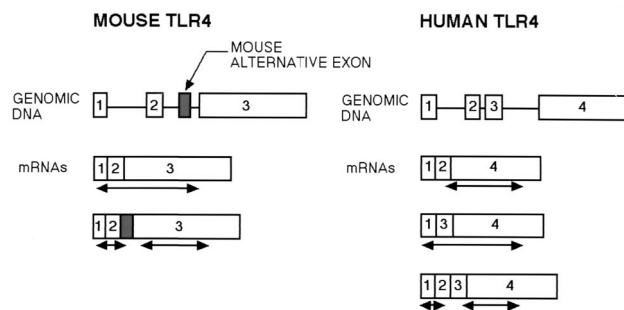


FIGURE 5. Comparison of the mouse and human *TLR4* gene structure. Exon/intron structure for mouse and human *TLR4* genes was schematically presented. Possible ORFs were indicated by bidirectional arrows.

ment (Fig. 2*B*). We have recently reported that TLR4 is expressed in T cells and may participate to promote Th1-type differentiation in response to LPS (5, 21). Our current data suggest that smTLR4 may also inhibit the excessive LPS responses in T cells.

We are not presently certain how LPS regulates this alternative splicing. Several regulatory mechanisms have been reported for the alternative splicing that include both positive regulation by specific factors such as TRA/TRA2 and negative regulation (exon skipping) in which splice sites are blocked by either protein factors or secondary RNA structure (reviewed by A. J. Lopez in Ref. 22). Alternative splicing is a powerful and versatile regulatory mechanism that can affect quantitative control gene expression and functional diversification of proteins. It is reported in virtually every type of protein including cell surface receptors (e.g., fibroblast growth factor receptor, Ref. 23; and α and β subunits of integrins, Ref. 24). The alternative splicing of mTLR4 resembles that of Sxl in *Drosophila* in that both of them regulate the expression of proteins by including or excluding stop codons (25). It also resembles that of IL-1 receptor accessory protein (IL-1R AcP) in that both are regulated in response to physiological cues and lead to the expression of soluble forms, which inhibit excessive cellular responses (26, 27).

We compared mouse and human *TLR4* genomic DNA (accession number AF177765) (Fig. 5). Mouse exons 1, 2, and 3 resemble human exons 1, 2, and 4, respectively. Thus, the location of the additional mouse exon studied here resembles that of the human third exon, although they do not show any obvious sequence homologies. Interestingly, the second or the third exon of the human *TLR4* gene is occasionally skipped like the additional mouse exon. In this alternative splicing, the second exon has an in-frame stop codon. Thus, various ORFs are possible according to the alternatively spliced mRNA isoforms (Fig. 5). Multiple mRNA isoforms are observed in the Northern blot analyses for both human and mouse *TLR4*, indicating that *TLR4* may be functionally regulated by alternative splicing in various species.

The incidence of infection with Gram-negative bacteria has increased in recent years especially among the immunocompromised patients, often causing serious endotoxin shock. All investigational therapies, including anti-TNF and anti-IL-1 agents, have uniformly failed to lower the mortality of critically ill patients with Gram-negative infection. Our data demonstrating that smTLR4 inhibits LPS-mediated TNF- α secretion, a major cause of endotoxin shock, may provide an insight into a new therapeutic approach controlling endotoxin shock.

Acknowledgments

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